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## **Studies on IL-15 mediated anti-tumour immune responses in Prostate Cancer**

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# **Studies on IL-15 mediated anti-tumour immune responses in Prostate Cancer**

**Christina Alexandra Sakellariou**

Submitted for the degree of Doctor of Philosophy in Immunology,  
in the Faculty of Life Sciences & Medicine

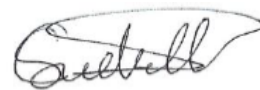
**June 2017**

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## Declaration of Thesis

I hereby declare that this Ph.D thesis entitled 'Studies on IL-15 mediated anti-tumour immune responses in Prostate Cancer' has been compiled by me under the supervision of Dr Christine Galustian, Professor Prokar Dasgupta and Dr Richard Smith, MRC Centre for Transplantation, King's College London. This thesis has not been previously submitted for the award of any degree at this or any other University or Institution.

Signed,

A handwritten signature in black ink, appearing to read 'Sakellariou', enclosed within a simple, hand-drawn oval border.

Christina Alexandra Sakellariou

June 12, 2017

## Abstract

The prostate cancer (PCa) microenvironment is profoundly immunosuppressive and infiltrating immune cells (e.g. CD8 T cells, NK cells) become anergic or suppressive. Immunosuppression is thought to be a key factor in PCa progression and the site of the cancerous prostate is where the immune system is most compromised.

We hypothesize that PCa can progress by tolerance to, and evasion of tumour cells from the immune system, and that therapies that act within the immunosuppressive PCa environment to reverse this immunosuppression are therefore desirable.

A coculture system of human non-adherent peripheral blood mononuclear cells (PBMCs) and PCa cell lines was set up, where IL-15 was found to be the most potent activator of NK and NKT cells, inducing the expansion of these cells, and the NK-mediated killing of the PCa cells. Other therapeutic Th1 cytokines had no effect within this environment.

Based on these results, the mechanisms of action of IL-15 on effector cell mediated killing of PCa cells in these coculture models were further investigated, by looking at its ability to modulate NK receptor and NK receptor ligand expression and interaction. It is shown that IL-15 enhances the NK cytotoxicity towards the PCa cells by upregulating the NKG2D receptor expression and downregulating both the surface and soluble expression of its ligand MICA.

A key focus of this project was to create novel immunotherapeutic agents that can be localised in the prostate and to determine their efficacy. Having identified IL-15 as a cytokine that uniquely activates NK cells within PCa-effector cell cocultures, the technique of cytotopic tailing was used; this enabled the modification of recombinant human IL-15 and two antibodies to immune checkpoint inhibitors into a cytotopic form, whereby they can attach to the cell membrane and exert their functions more efficiently and for a prolonged period of time. The activity and function of tailed IL-15 and of the two tailed antibodies were tested *in vitro* as well as in an *in vivo* tumour challenge model of C57BL/6 mice injected with TRAMP-C2 tumour cells. Complete tumour clearance was observed in mice that were treated with the tailed cocktail of drugs, unlike the untail version of this cocktail or the control.



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## Abbreviations

ADCC	-	Antibody - dependent Cellular Cytotoxicity
AML	-	Acute Myeloid Leukaemia
aNKR	-	Activatory Natural Killer Receptor
APC	-	Antigen Presenting Cell
BSA	-	Bovine Serum Albumin
CCL2	-	C-C motif chemokine ligand 2
CCL5	-	C-C motif chemokine ligand 5
CD	-	Cluster of Differentiation
CPRC	-	Castrate-resistant Prostate Cancer
CR1	-	Complement Receptor 1
CTLA-4	-	Cytotoxic T Lymphocyte Antigen- 4
DC	-	Dendritic Cell
DMEM	-	Dulbecco's modified Eagle Medium
DMSO	-	Dimethyl Sulfoxide
DNAM-1-	-	DNAX accessory molecule 1
DRE	-	Digital Rectal Exam
ELISA	-	Enzyme- linked Immunosorbent Assay
ERK	-	Extracellular Signal-Regulated Kinase
FACS	-	Fluorescence Activated Cell Sorting
FBS	-	Foetal Bovine Serum
FDA	-	Food and Drug Administration
FoxP3	-	Forkhead Box P3
GM-CSF-	-	Granulocyte Monocyte Colony- Stimulating Factor
GVAX	-	Granulocyte-Macrophage Colony-Stimulating factor Tumour Cell Vaccine
H&E	-	Haematoxylin & Eosin
HBSS	-	Hank's Balanced Salt Solution
HLA	-	Human Leukocyte Antigen
IDO	-	Indolamine-2,3-Dioxygenase
IFN	-	Interferon

Ig	-	Immunoglobulin
IL	-	Interleukin
iNKR	-	Inhibitory Natural Killer Receptor
ITAM	-	Immune Tyrosine-based Activating Motifs
ITIM	-	Immunoreceptor Tyrosine-based Inhibitory Motifs
KIR	-	Killer immunoglobulin- like Receptor
Ly46	-	Lymphocyte Antigen 46
MHC	-	Major Histocompatibility Complex
MMP	-	Matrix Metalloproteinase
MTS	-	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium)
NCR	-	Natural Cytotoxicity Receptors
NK	-	Natural Killer
NKG2	-	Natural Killer Group 2
NKR	-	Natural Killer Receptor
NKT	-	Natural Killer T
NK- $\kappa$ B	-	Nuclear factor Kappa-light-chain-enhancer of Activated B Cells
NOS	-	Nitric Oxide Species
PAMP	-	Pathogen Associated Molecular Pathogens
PAP	-	Prostatic Acid Phosphatase
PBMCs	-	Peripheral Blood Mononuclear Cells
PCa	-	Prostate Cancer
PD-1	-	Programmed Death - 1
PD-L1	-	Programmed Death Ligand-1
PGE2	-	Prostaglandin E2
PHA	-	Phytohemmagglutinin
PI	-	Propidium Iodide
PI3K	-	Phospholinositide-3 Kinase
PBS	-	Phosphate Buffered Saline
PRR	-	Pattern Recognition Receptors
PSA	-	Prostatic Acid Phosphatase
RCC	-	Renal Cell Carcinoma
ROS	-	Reactive Oxygen Species

RPMI	-	Roswell Park Memorial Institute Medium
SDS-PAGE-		Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
SHP-1	-	Src homology region 2 domain-containing phosphatase-1
SHP-2	-	Src homology region 2 domain-containing phosphatase-2
Syk	-	Spleen tyrosine kinase
TAA	-	Tumour Associated Antigen
TCR	-	T Cell Receptor
TGFβ	-	Tumour Growth Factor- β
Th1	-	T helper 1
Th2	-	T helper 2
Th17	-	T helper 17
TIL	-	Tumour Infiltrating Lymphocyte
TIM3	-	T cell Immunoglobulin domain and Mucin domain-containing molecule 3
TNFα	-	Tumour Necrosis Factor α
TRAIL	-	TNF-related apoptosis inducing ligand
Treg	-	T regulatory
ULBP1	-	UL-16 Binding Protein-1
VEGF	-	Vascular Endothelial Growth Factor
VITAL	-	Vaccine Immunotherapy with Allogeneic Prostate Cancer Cell Lines
ZAP-70-		Zeta chain of T cell receptor Associated Protein kinase 70
γδ	-	gamma delta

## CHAPTER 1

### Introduction

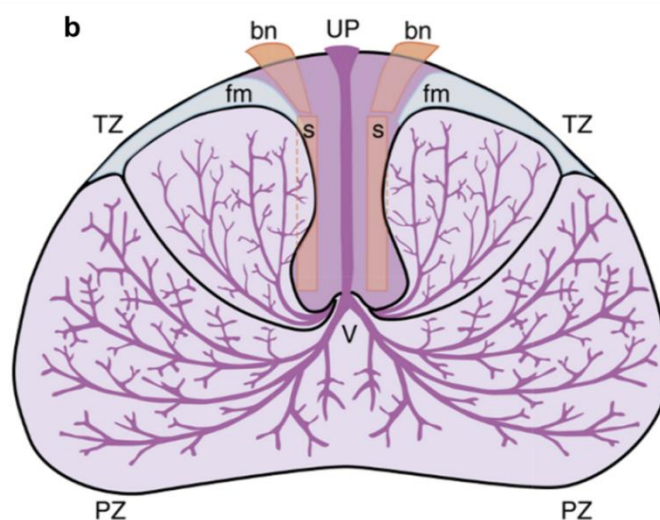
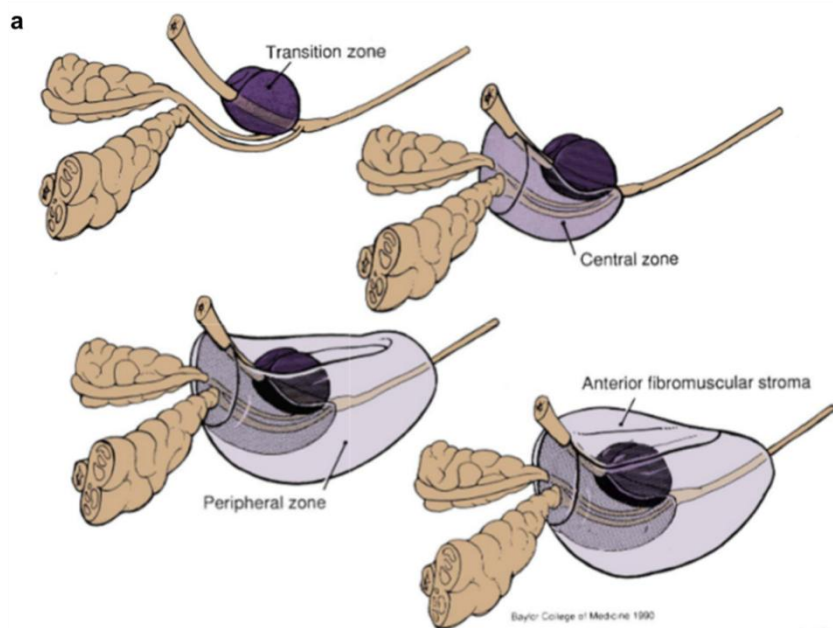
## **1.1 Nature of cancer and epidemiology**

The introduction of vaccination, antibiotics and other preventive measures have clearly contributed to the reduction of the mortality rates due to infectious diseases during the last century, while the incidence of cancer has dramatically increased, making it a global health issue (Jemal, Bray et al. 2011). Cancer is a disease defined by the uncontrollable replication of cells, caused by altered genes. Those genes are functionally categorised into two groups, oncogenes and tumour suppressor genes (Hanahan and Weinberg 2011).

In the UK, around 300,000 new cases of cancer were registered in 2014, of which most were in males; out of 200 different types of cancer, breast, lung, colorectal and prostate were the most common, accounting for more than 50% of all cancer cases (Murphy 2016, Siegel, Miller et al. 2016).

### **1.1.1 The prostate**

The adult prostate resembles the shape of a walnut and weighs around 18g (Wein 2012). It is a firm rubbery gland, located at the base of the bladder, surrounding the posterior urethra. It is situated posterior and inferior to the symphysis pubis, superior to the urogenital diaphragm and anterior to the rectal ampulla (Figure 1.1). McNeal has described several different zones of the prostate. This model is still used in the contemporary literature with some modifications: a) the central zone around the urethra which forms 5-10% of the glandular tissue in young men, b) the transition zone that can enlarge later in life and cause bladder outflow symptoms, c) the peripheral zone which comprises 75% of glandular volume in the adult prostate and where majority of prostate cancers have been shown to arise. Another zone is d) the anterior fibromuscular stroma which has little significance generally and contains little glandular tissue. The main tissue types that form the prostate gland are the epithelial tissue, containing secretory glandular cells, non-secretory basal cells and neuroendocrine cells and the stromal compartment containing various cell populations, such as fibroblasts, lymphocytes and others (McNeal 1981, Wein 2012).



**Figure 1.1: Anatomy of the prostate: a)** McNeil's four different zones of the prostate are shown. The transition zone lies proximal to the ejaculatory ducts and ventral and dorsal to urethra. The central zone surrounds the ejaculatory ducts. The peripheral zone constitutes the bulk of the apical, posterior and lateral aspects of the prostate. The anterior fibromuscular zone covers the transition zone and the distal urethra ventrally. Photo adapted from Hinman's Atlas of Urosurgical Anatomy (MacLennan 2012) **b)** Diagram showing a coronal section of the prostate. PZ: peripheral zone, TZ: transition zone, UP: proximal urethral segment and periurethral glands, V: verumontanum, s: preprostatic sphincter, bn: bladder neck. Adapted from (McNeal J.E. 1990).

### 1.1.2 Background to Prostate cancer: Epidemiology and Treatment

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second most common type of cancer causing death in men, after the first killer which is lung cancer, in the UK. Every year, more than 45,000 men are diagnosed with PCa and it is thought that 1 in 8 men will develop it at some point in their lives. In Europe, PCa is the third most common cause of cancer and the sixth most common cause of cancer death overall (McLaren 2016, Murphy 2016).

PCa is a generally slowly progressing disease, which over time could metastasize, as the cancer cells develop alterations in their shape and in their attachment to other cells and to the extracellular matrix, losing the expression of adherence molecules (e.g. E-cadherin) and undergoing epithelial- mesenchymal transition (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). The most significant factor for developing the cancer is age (75% of PCa cases are diagnosed in men over the age of 65) (Siegel, Naishadham et al. 2012), since aging is associated with a number of physiological changes, such as faulty DNA repair mechanisms, immunosenescence and alterations in hormone levels. These changes aid the structural remodelling of prostate cells leading to modifications in gene expression, which affect cellular processes, such as apoptosis and proliferation. These result in stromal changes in the prostate gland, enhancing the tumour growth, and as the cancerous prostate is very immunosuppressive the tumour progresses further (Barron and Rowley 2012).

Prostate carcinomas express a range of cell markers, including androgen receptors and protein specific antigen (PSA). The latter is present in small quantities in the serum of healthy men. An elevation of PSA levels should be investigated, hence the PSA testing in addition to a Digital Rectal Examination (DRE) are the early PCa screening tools currently used. Definitive diagnosis is done by histopathological verification on a prostate biopsy, except in the case of a clearly abnormal DRE, or of metastasis on a bone scan or of a very high PSA; then the histology is often not needed.

Prostate cancer grading system uses Gleason scoring (GS) as it was developed by Donald Gleason. This system assigns each histological sample with a pattern ranging from 1 to 5 and the addition of the most prevalent and the second most common scores, provides the grade



which could range between 2 and 10. To date, the Gleason grading system has been revised twice, in 2005 and 2014; however it has presented many weaknesses that impacted on patient care, and as it seemed critical to design a new grading system Epstein *et al* in 2013, proposed a more accurate and simplified grading system. In that, five grade groups were presented, based on the histological examination of the samples: "Grade group 1 (GS 3+3=6) was given to individuals with discrete well-formed glands; group 2 (GS 3+4=7) was given to those with predominantly well-formed glands with lesser component of poorly formed/fused/cribriform glands; group 3 (GS 4+3=7), to those with predominantly poorly formed/fused/cribriform glands with lesser component of well-formed glands; group 4 (GS 8) to those with either poorly formed/fused/cribriform glands, or predominantly well-formed glands and lesser component lacking glands, or predominantly lacking glands and lesser component of well-formed glands; group 5 (GS 9-10) to those with lack of gland formation or with necrosis, with or without poorly formed/fused/cribriform glands" (Epstein, Zelefsky et al. 2016).

There are several different types of treatment options available for PCa patients, depending on a system of risk stratification (that takes into account the PSA level, rectal exam finding, histological grade of the tumour, Gleason scoring) in addition to patient's age, comorbidities and patient preference. The staging system that is used to date is the TNM (tumour, nodes, metastasis) system by the American Joint Committee on Cancer (AJCC). It classifies the tumour from grade I to IV, but also sub-categorises it, dependent on whether the lymph nodes are affected, the extent and also the presence of metastasis. Treatment options are based on this staging system (Edge, Byrd et al. 2010). Some men with localised PCa are candidates for deferred management. Active surveillance is an emerging and increasingly used option. This is mainly used to reduce overtreatment in patients with clinically confined low-risk PCa. However, radical prostatectomy, brachytherapy and radiotherapy are currently the treatment options available with curative intent for localised PCa. Once the tumour has progressed locally, within the prostate microenvironment, a routine treatment method is radiotherapy or watchful waiting. This option differs from active surveillance, as in watchful waiting the treatment is deferred till symptoms develop and when they do, the treatment is symptomatic control. If the tumour locally advances, hormone therapy could be additionally introduced to the patient in combination with radiotherapy. Hormone therapy alone could be suggested only if the cancer has spread to

another part of the body (metastatic disease). It works by blocking androgen receptors (gonadal and adrenal) that complement testosterone. The PCa growth is dependent on androgens as it had been shown that mice deprived of androgens, showed a regression of their prostate tumours (Heinlein and Chang 2002). Androgen receptor signalling regulates the growth and development of the prostate and maintains prostate homeostasis (Heinlein and Chang 2002). However, if the anti-androgen therapy, followed by luteinizing hormone-releasing hormone (LHRH) analogue monotherapy or combination of the two, becomes ineffective and the PSA keeps rising with the tumour becoming castrate resistant, then chemotherapy is considered as the next line of treatment (Mottet 2015).

All of these current treatments have considerable side effects: Surgery can render patients incontinent and/or impotent, while radiotherapy and chemotherapies could additionally cause fatigue, hair loss, nausea, neutropenia and anaemia, due to low numbers of white and red blood cells, and can destroy normal tissue as well as cancer cells (Dearnaley, Khoo et al. 1999). Due to the unpleasant side effects of these therapies, immunotherapy of PCa has been put forward as an alternative modality. Unfortunately, immunotherapy is introduced to the patient, once the cancer has metastasized and the patient has already undergone hormone therapy and chemotherapy, therefore the immune system is exhausted. This creates a great need for the development of novel therapies, which will offer a more personalised and tailored approach to patients who are refractory to the other available types of treatments.

## **1.2 Immunity and cancer**

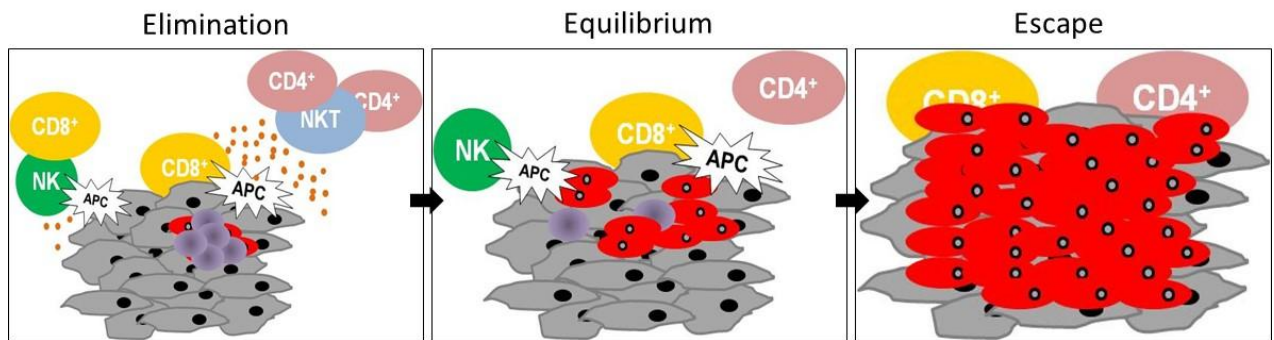
Cancer progresses by a combination of the ability of the cancer cell to transition and acquire mesenchymal properties and ability to metastasize, in addition to the failure of the body's biological mechanisms to recognise and eliminate rogue cells. When the immune system fails to activate the anti-tumour immunity, cells begin to replicate uncontrollably, transforming, outgrowing and becoming neoplasms and eventually invasive tumours.

### **1.2.1 The concept of immunoediting**

Paul Ehrlich introduced the idea of immunotherapy more than 100 years ago, by suggesting that the immune system can attack transformed cells, such as tumour cells (Ehrlich 1906). It was believed that the host and tumour interactions are manipulated to favour targeting and the elimination of the tumour cells by the host immune cells, without harm to normal tissue.

Since then, the concept of cancer immunoediting has become a central concept. This entails three main phases; elimination, equilibrium and escape.

During the elimination phase, the immune system recognises the tumour cells and destroys them through innate and adaptive immune mechanisms. Initially innate immune cells like macrophages recognise the tumour cells and carry out limited killing. However as the tumour grows, stromal remodelling occurs and inflammatory cytokines are secreted by the surrounding macrophages, stromal and tumour cells leading to the recruitment of other innate immune effector cells such as Natural Killer (NK) cells and Dendritic Cells (DC) which attack the tumour cells. The DCs, which are a type of Antigen Presenting Cells (APCs), present the tumour antigens to CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the context of MHC class II and class I molecules, respectively, and consequently the CD8<sup>+</sup> T cells move to the tumour site to target and kill the tumour cells (Dunn, Old et al. 2004, Inoue, Yamazaki et al. 2006). However, the elimination phase creates a poorly immunogenic environment, due to the induction of new tumour variants that are resistant to immune effector cells, therefore an equilibrium phase is reached. This phase could occur over many years, where there is a balance between the new tumour cells that arise and the immune system trying to destroy them. Eventually, the tumour cells develop enough mutations, conferring resistance to the immune system's 'attack' which allows tumour evasion and an escape phase is established (Dunn, Koebel et al. 2006). At that point tumour cells proliferate uncontrollably, resulting in the development of clinically detectable cancer (Figure 1.2).



**Figure 1.2: Illustration of the cancer immunoediting processes.** At the beginning of the immunoediting process, the immune system cells eliminate the tumour cells, however tumour cell variants may become resistant to immune cell targeting and may start proliferating uncontrollably due to selection pressure, thus creating resistance and escaping from the immune cell system attack.

## 1.2.2 Types of immune effector cells involved in the anti-tumour immune response

There are several immune effector cells of both the innate and of the adaptive immune system. These effector cells are thought to be able to recognise cancer cells as foreign and destroy them. These include NK cells, NKT cells,  $\gamma\delta$  T cells, CD8 T helper cells and DCs.

### 1.2.2.1. NK and NKT cells

NK cells are considered part of the innate immune system, and comprise 2-18% of human peripheral blood lymphocytes (Vivier, Tomasello et al. 2008). They are largely found in the spleen and in lymph nodes (Witte, Wordelmann et al. 1990). They were discovered in the 1970s and were described as large granular lymphocytes (Cerwenka and Lanier 2001). They are the first line of defence against an infection or a pathogen, capable of killing cells infected with viruses. In addition NK cells are able to kill mutated cells or tumour cells; it has been shown that NK cells are capable of killing virus-induced leukaemia cells and they are able to achieve this killing without the need of prior sensitisation to the target cells (Kiessling, Klein et al. 1975, Herberman and Ortaldo 1981, Lanier 2005).

It is important that NK cells are able to distinguish between healthy, stressed and transformed cells, so they have developed several mechanisms by which they recognise healthy cells that should not be targeted and those that should be attacked. These mechanisms form the basis of NK cell activation (Lanier 2005). They are activated either via their receptors, by which they can lyse cells without prior antigen stimulation, or via antibody- dependent cellular cytotoxicity (ADCC), which is exerted upon binding of the FcγRIIIα (CD16), expressed by NK cells, to the Fc part of the antibody-coated target cells causing the phosphorylation of immune tyrosine-based activating motifs (ITAM). This leads to binding to tyrosine kinases ZAP-70 and Syk, activation of PI3K, NF-κB and ERK pathways, and cytokine secretion. This ultimately leads to lysis of target cells (Seidel, Schlegel et al. 2013, Wang, Erbe et al. 2015). CD16 has been also shown to play a direct role in NK cell cytotoxicity, independent of antibody binding (Mandelboim, Malik et al. 1999) and it has been reported that CD16<sup>dim</sup> CD56<sup>bright</sup> NK cells have increased cytokine production, but it is the CD16<sup>bright</sup> CD56<sup>dim</sup> populations that show an increased cytotoxic capacity (Vivier, Tomasello et al. 2008). NK cells can be primed in a DC-dependent way, in response to various cytokines, such as IL-2, type I IFNs and IL-15 (Walzer, Dalod et al. 2005).

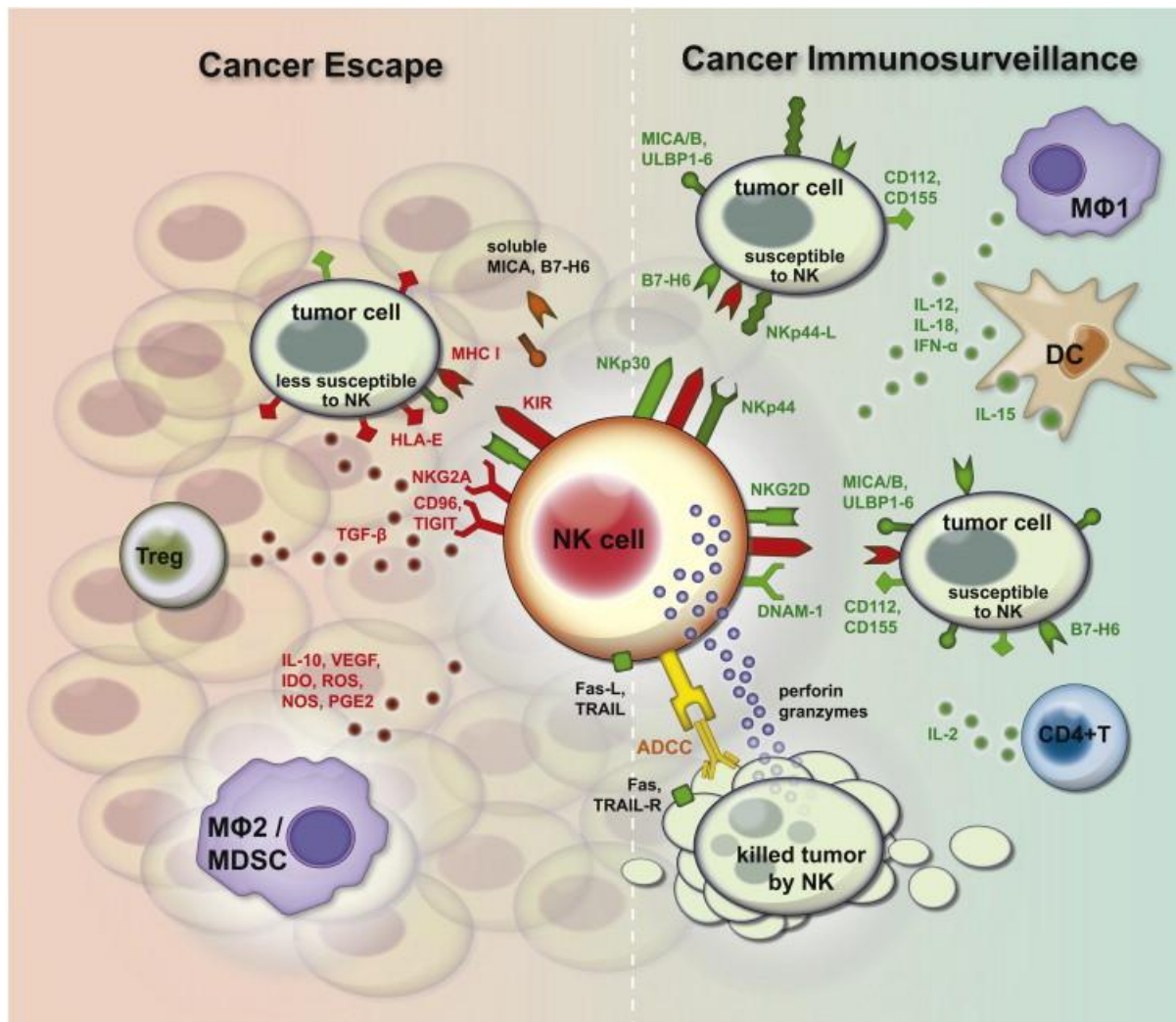
NK cells are regulated and have the ability to distinguish transformed cells from healthy cells, by two recognition pathway mechanisms called ‘missing-self’ and ‘induced-self’ (Karre 2002, Bryceson, March et al. 2006). The ‘missing-self’ mechanism is led by the lack of protection of self-cells and involves the recognition of an altered stress-induced glycoprotein cell surface composition, together with an ‘induced-self’ MHC molecule expression change, through an array of inhibitory and activatory receptors that are expressed on their cell surface.

The inhibitory receptors (iNKR) that they express, recognise molecules of the major histocompatibility complex- I (MHC-I); for humans these receptors are called killer immunoglobulin-like (KIR) and in mice Ly46; additionally they both express a heterodimer of CD94-NKG2A. As tumour cells express low levels of MHC-I molecules, they often escape this inhibitory signalling by NK cells, so in order for NK cells to be activated, they rely on the stimulatory signals by the activatory NK receptors (aNKR), which recognise stress-induced signals on the surface of the target cells (Bottino, Castriconi et al. 2005). Notable examples

are NKG2D and DNAM-1 as well as the natural cytotoxicity receptors NKp30, NKp44 and NKp46. The NK receptors and ligands will be further discussed on section 1.2.3.

Cell killing is mediated through the release of cytotoxic granules containing perforin and granzyme (Voskoboinik, Smyth et al. 2006, Topham and Hewitt 2009), the ligation of death receptors such as TRAIL and Fas Ligand and through the release of a number of pro-inflammatory cytokines and chemokines, such as IFN $\gamma$ , TNF $\alpha$ , GM-CSF, CCL2 and CCL5 which are involved in the activation of the adaptive immune system cell and which will further attack the tumour cells (Yokoyama, Kim et al. 2004, Zwirner and Domaica 2010, Bernardini, Gismondi et al. 2012, Romee, Leong et al. 2014). Hence, it is believed that NK cells have also immunomodulatory properties, as they can positively or negatively influence anti-cancer responses by DCs and T cells (Sungur and Murphy 2014).

The important role of NK cells in immunosurveillance was supported by a study in mice where it was shown that RAG $^{-/-}$  mice (lacking adaptive immunity) had decreased tumour incidence and formation, compared to RAG $^{-/-}$  x  $\gamma$ c $^{-/-}$  (lacking all lymphocytes, both of the innate and adaptive immunity) (O'Sullivan, Saddawi-Konefka et al. 2012). Furthermore, Guerra *et al.* 2008 showed that in a TRAMP mouse model, NKG2D-deficient mice (Klrk1 $^{-/-}$ ) were three times more susceptible than wild-type mice to developing highly malignant tumours, demonstrating the important role of the NK-NKG2D interaction in immunosurveillance (Guerra, Tan et al. 2008). Figure 1.3 is adapted by (Pahl and Cerwenka 2015) and shows the regulation of NK cells during cancer immunosurveillance and cancer escape.



**Figure 1.3: NK cell cytotoxicity regulation during cancer immunosurveillance and cancer escape.** NK cells interact with cancer cells via the inhibitory (in red) and the activatory (in green) receptors and their corresponding ligands on tumour cells. Target cell apoptosis is induced via perforin and granzyme production, through the Fas Ligand and TRAIL expressed on NK cells and via CD16 antibody-dependent cellular cytotoxicity (ADCC). The involvement of cytokines (e.g. IL-18, IL-2, IL-15) produced by immune effector cells stimulate the NK cell cytotoxicity. TGF $\beta$ , IL-10, prostaglandin E2 (PGE<sub>2</sub>), vascular endothelial growth factor (VEGF), nitric oxide species (NOS) and reactive oxygen species (ROS), produced by immunosuppressive cell populations (e.g. Treg, Macrophages) promote the tumor escape from NK cell surveillance and the immunosuppression in the cancer microenvironment. Adapted from (Pahl and Cerwenka 2015).

NKT cells comprise about 1% of circulating lymphocytes and they differ from NK cells because of the antigen-specific T cell receptor (TCR) that they express on their surface. NKT cells express the CD56 marker as NK cells, and also CD3 as T cells (Godfrey, MacDonald et al. 2004).

NKT cells are thought to create a bridge between the innate and the adaptive immunity and they are classified into two categories –Type I and Type II- dependent on their role in immunity. Type I NKT cells express an invariant V $\alpha$ 14J $\alpha$ 18 TCR whereas type II express variable TCRs (Cardell, Tangri et al. 1995). Both types produce Th1 and Th2 cytokines, IFN $\gamma$  and IL-4 respectively, upon stimulation with the CD1d antigen presentation. Type I NKT cells have a protective role dependent on their ability to produce IFN $\gamma$ , however they don't act directly on tumour cells, although they are able to lyse cells. Rather, Type II NKT cells act through their effects on other types of immune cells such as NK and CD8 T cells, or DCs, which make IL-12 thus contributing to their anti-tumour effect (Toura, Kawano et al. 1999, Metelitsa, Naidenko et al. 2001). Quite paradoxically, type II NKT cells are thought to be involved in the suppression of the anti-tumour response (O'Konek, Kato et al. 2013).

#### 1.2.2.2 CD8 T cells

Cytotoxic T cells (CD8 T cells), an adaptive immune cell type, become activated and induce cytolysis and IFN $\gamma$  production, upon their encounter with aberrant antigens bound to MHC Class I molecules, presented by the Antigen Presenting Cell (APC).

Many immunotherapeutic strategies are designed to stimulate and enhance the CD8 T cell activity by targeting and overcoming the peripheral tolerance, which exists to protect healthy tissues from immune mediated attack, since tumours also express self-antigens characteristic of the healthy tissues from which they are derived.

It has been suggested that intraprostatic CD8<sup>+</sup> T cells are non-functional, or unable to upregulate activation markers such as CD69 or CD137 in response to external stimuli (PMA/ionomycin) (Bronte, Kasic et al. 2005). Furthermore, the secretion of TGF $\beta$  and IL-10 by tumour cells has been shown to confer a regulatory CD8 effector T cell phenotype within the PCa microenvironment whereby FoxP3 may be expressed by these cells, enabling them to suppress any CD8 effector cells and therefore their cytotoxic activity towards tumour cells;



this may be one of the mechanisms that tumour cells escape the immune response (Shafer-Weaver, Anderson et al. 2009).

### **1.2.2.3 Gamma delta ( $\gamma\delta$ ) T cells**

$\gamma\delta$  T cells are a small proportion of circulating T lymphocytes (1-5%) , that express antigen receptors that undergo VDJ (variable-diversity-joining) recombination creating a repertoire diversity in T cells. They are mostly abundant in mucosal sites and they contribute to immune response, infection and tissue damage, by producing pro-inflammatory cytokines such as IFN $\gamma$  and IL-17 as well as anti-inflammatory cytokines like TGF $\beta$  and IL-10 (Rincon-Orozco, Kunzmann et al. 2005, Kuhl, Pawlowski et al. 2009, Rei, Goncalves-Sousa et al. 2014). They have been reported to have a protective role in B cell lymphomas and in PCa, due to the IFN $\gamma$  production, however, it has been shown that they can also promote tumour growth due to the IL-17 production (Rei, Pennington et al. 2015).

### **1.2.2.4 Macrophages and Dendritic cells (DCs)**

Macrophages and DCs are innate immune cells that are activated through cellular receptors known as pattern recognition receptors (PRRs). These are activated through pathogen-associated molecular patterns (PAMPs), which are present on a variety of microorganisms, such as bacteria, fungi and viruses, but are not present on tumour cells. So PRRs do not become activated to attack cells expressing tumour-associated antigens (TAA) and as a consequence the innate immune cells fail to elicit a response (Berke 1994).

DCs are bone marrow-derived cells that provide a link between innate and adaptive immunity, as they control both tolerance and immune responses (Reis e Sousa 2001). Immature DCs recognise antigens either a) by phagocytosis, using complement receptors and Fc receptors and C-type lectins, or b) by micropinocytosis, or c) by using signalling receptors, such as TLRs, that recognise PAMPs, and respond by secreting cytokines (eg. IL-12, IFN $\alpha$ ). It is believed that unlike other APCs (e.g. Macrophages, B cells), their only function is to stimulate the adaptive immunity by presenting the foreign antigens in a complex of peptide-MHC I or II

molecules to naïve CD8 or CD4 T cells respectively, which activates the T cells, especially the cytotoxic CD8<sup>+</sup> T cells to act against the target cells (Palucka and Banchereau 2012).

### 1.2.3 Mechanism of tumour cell killing by NK cells: NK receptors and ligands

NK cells have developed mechanisms that aid their activation in order to effectively distinguish between healthy and tumour cells and eventually cause target lysis. They do that either via ADCC or via the interaction between the NK receptors (NKR) and their corresponding ligands on tumour cells.

The human KIR inhibitory receptor genes demonstrate extensive polymorphism and encode for type I transmembrane glycoproteins that evolved from the immunoglobulin (Ig) superfamily, having two or three Ig-like domains (designated as KIR2D- or KIR3D-) in the extracellular region, which have been designated as D0, D1 or D2. Within the KIR subfamilies heterogeneity is observed in terms of the cytoplasmic domain, which could be either long (designated as KIR2DL- or KIR3DL-), characterised by two or three immunoreceptor tyrosine-based inhibitory motifs (ITIM) or short (designated as KIR2DS- or KIR3DS-), lacking ITIMs (Colonna and Samaridis 1995).

ITIMs are found in the cytoplasmic domains of all inhibitory NKRs, with a consensus sequence Ile/Val/Leu/Ser-x-Tyr-x-x-Leu/Val, where x denotes any amino acid. Upon ligand binding, tyrosine phosphorylation occurs by a src kinase that leads to an NK cytotoxicity or cytokine production, via the recruitment of tyrosine-specific phosphatases SHP1 or SHP2 or the phospholipid-specific phosphatase SHIP (Muta, Kurosaki et al. 1994, Binstadt, Brumbaugh et al. 1996).

KIRs recognise ligands of the HLA protein family (A, B or C) and can discriminate between different peptides presented by them (Colonna, Spies et al. 1992) (see table 1.1). However, the biological relevance is still unclear, as these KIR receptors do not distinguish between self and non-self peptides.

The activatory receptors use adapter molecules containing an immunoreceptor tyrosine-based activation motif (ITAM) to provide signalling function. These ITAM motifs have a consensus sequence of Asp/Glu-x-x-Tyr-x-x-Leu/Ile x<sub>6-8</sub> Tyr xx Leu/Ile, where x represents

any amino acid, with 6 to 8 amino acids between tyrosine (Tyr) and Leucine/Isoleucine (Leu/Ile). Upon phosphorylation of the ITAM, the tyrosine kinases Syk and Zap70 (zeta chain of T cell receptor associated protein kinase 70) are recruited and activate downstream events that lead to NK receptor signal transduction (Reth 1989).

One of the most well investigated activatory receptors is NKG2D, member of the NKG2 family of genes, which however has very little homology to the other member of the family. It is a type II transmembrane-anchored glycoprotein, expressed in humans by NK cells as well as by CD8<sup>+</sup> T cells. NKG2D lacks an ITAM motif, therefore adapter molecules like Dap10 are needed for signal transduction (via the PI3K pathway) that will trigger cytotoxicity (Wu, Song et al. 1999).

NKG2D recognises ligands of the MHC Class I family and in humans these are MICA and MICB, induced upon cellular stress, and the UL-16 Binding Proteins 1-4 (ULBP1-4) (Bauer, Groh et al. 1999). Tumour cells exploit these ligands by shedding them from the cell surface, in order to evade the immune system and escape the immune surveillance of NK cells (Waldhauer and Steinle 2006, Waldhauer, Goehlsdorf et al. 2008).

Another family of activatory receptors is that of the Natural Cytotoxicity Receptors (NCRs). There are three members of that family, NCR1 (NKp46), NCR2 (NKp44) and NCR3 (NKp30), which are exclusively expressed on NK cells, but NKp44 is expressed only by activated NK cells. Phosphorylation of their ITAM motifs activates signalling pathways that leads to cytotoxicity and cytokine degranulation (Sivori, Vitale et al. 1997, Vitale, Bottino et al. 1998, Pende, Parolini et al. 1999). To date only a few of their ligands have been identified. Table 1.1 shows a selected panel of receptors and their corresponding ligands that were further investigated in this project.

Receptor	Ligand	Function
Killer Immunoglobulin-like receptors (KIRs)		
KIR2DL1	HLA-C, C2	Inhibitory
KIR2DL2/3	HLA-C, C2	Inhibitory
KIR3DL1	HLA-A, HLA-B Bw4	Inhibitory
Cytotoxicity receptors		
NKp30	Unknown; B7-H6 homolog?	Activatory
NKp44	NKp44L- isoform of the mixed-lineage leukemia-5 (MML5) protein	Activatory
NKp46	Unknown	Activatory
NKG2D	MICA, MICB, ULBP family proteins	Activatory
DNAM-1	Nectin-2	Activatory

Table 1.1: A selected panel of activatory and inhibitory NKRs and their corresponding ligands that are investigated in this project.

## 1.3 Immunosuppression in the PCa microenvironment

### 1.3.1 Suppressive immune cell populations and proteins expressed within the cancerous prostate and associated stroma

In recent years, there has been increasing evidence that immunosuppression associated with a lack of activity of immune effector cells such as NK cells contributes towards progression of PCa (Koo, Shim et al. 2013). The PCa microenvironment is highly immunosuppressive and it is suggested that although immune cells (e.g. NK and CD8 Tcells) infiltrate the cancerous prostate, they are rendered anergic or even regulatory (Shafer-Weaver, Anderson et al. 2009).

The immune cell types that infiltrate the prostate have been demonstrated in several studies. These include the IFN $\gamma$ -producing Th1 cells, IL-4-producing Th2 cells and IL-17-producing Th17 cells, as well as CD4<sup>+</sup>, CD8<sup>+</sup> and FoxP3<sup>+</sup> Treg cells (Kiniwa, Miyahara et al. 2007, Sfanos, Bruno et al. 2008). In a separate study by Hadaschik *et al.* (2012), T cells isolated from peripheral blood of PCa patients showed an increased response rate to TAA compared to those isolated from benign prostatic hyperplasia (BPH) patients or healthy donors. Furthermore, they showed an increased T cell response when Treg cells were depleted, demonstrating a suppressive phenotype of these Treg cells towards the immune response in PCa patients (Hadaschik, Su et al. 2012). Using the TRAMP mouse model of PCa, it was observed that although T-antigen specific T and B cells were capable of recognising tumour cells, the cytotoxic T lymphocytes were unable to kill them due to the immunosuppression caused in the tumour microenvironment (Willimsky, Czeh et al. 2008).

When trying to understand the mechanisms of immunosuppression in the PCa microenvironment, a number of immunosuppressive factors in the form of either secreted or cell surface proteins, growth factors or regulatory immune cells were shown to contribute. In the study by Kiniwa *et al.* (2007) that was mentioned earlier, an increased number of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> Treg cells in the prostate tumours was observed, however in addition to these, another rarer population of CD8<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> seemed to enter the cancerous prostate and suppress both the T cell proliferation and activity and NK cell function (Kiniwa, Miyahara et al.

2007). In further studies, the rare population of T suppressor cells ( $CD8^+ CD25^+ FoxP3^+$ ) was found to mediate and suppress T- effector cell responses to TAA, through CTLA-4 (Cytotoxic T Lymphocyte Antigen-4) expression and IL-35; CTLA-4 blockade relieved the suppressive phenotype (Olson, Jankowska-Gan et al. 2012, Arruvito, Payaslian et al. 2014). Yu *et al.* 2012, also demonstrated that CTLA-4 and PD-L1 (Programmed Death Ligand-1) blockade 'break' the Treg suppression of IL-15-induced CTL immunity in TRAMP tumours in mice (Yu, Steel et al. 2012).

Regarding NK cells, which are the main focus in this study, a defect in their cytotoxic activity as PCa progresses to the metastatic stage has been observed (Koo, Shim et al. 2013, Liu, Lu et al. 2013), which is attributed to the presence of cancerous cells in the circulation (Kastelan, Kraljic et al. 1992). Another type of cells that infiltrate the PCa environment and have a suppressive function against NK cells, T cells and DCs, are the immature myeloid suppressor cells (Almand, Clark et al. 2001, Gabrilovich, Ostrand-Rosenberg et al. 2012). These are either recruited by tumour cells or they are formed from active non-suppressive cell populations through cytokine secretion by tumour or stromal tissue (Elhage, Galustian et al. 2012).

Moreover, Treg cells seemed to suppress the NK cell function in a  $TGF\beta$  dependent manner, through the downregulation of NKG2D both *in vitro* and *in vivo* in a study by Ghiringelli *et al.* (2005); blockade of Treg cells ameliorated the NK cell cytotoxic activity (Ghiringelli, Menard et al. 2005).

$TGF\beta$  is a growth factor that has been implicated in the tumour immunosuppression in PCa. Many tumours produce high levels of  $TGF\beta$ , hence its blockade or impairment could enhance the tumour rejection by the immune system. A suggested mechanism by which tumour cells evade the immune system is through the conversion of  $CD4^+$  T cells into Treg cells in the tumour sites due to the  $TGF\beta$  that is produced in the tumour cells (Liu, Wong et al. 2007).

Together with IL-10, they are both secreted by fibroblasts and cells surrounding the PCa environment, promoting angiogenesis and inhibition of the CD4 and CD8 T cell proliferation (Ahuja, Paliogianni et al. 1993, Arteaga, Hurd et al. 1993).

Two separate studies by Zhang *et al.* (2006) and Shafer-Weaver *et al.* (2009) showed that  $TGF\beta$  signalling blockade induced CTL tumour infiltration in mice, where CD8 T cells specific for the tumour were adoptively transferred (Zhang, Yang et al. 2006, Shafer-Weaver,

Anderson et al. 2009). However, other studies have suggested that long-term TGF $\beta$  blockade could result in autoimmunity, hence a fine balance between tumour immunity and autoimmunity should be maintained.

A number of cell surface and secreted proteins are expressed by cells of the tumour microenvironment which can inhibit the immune response by effector immune cells: these include complement regulatory proteins, such as CD46, CD59, CD55 and CD97 which are highly expressed in tumour cell populations (Loberg, Wojno et al. 2005) and inhibitory ligands of the MHC family that inactivate NK, CD8 and dendritic cells (Lanier and Phillips 1996); also, a number of cytokines and chemokines that are thought to be secreted by tumour stromal cells (in addition to their normal production by haemopoietic cells). Instead of activating the infiltrating immune cells, they inhibit them through their aberrant structures, locations and presentation on the cells. In a study by Su *et al.* (2010) it was reported that tumour-derived fibroblasts secrete MCP-1 (CCL2) and RANTES (CCL5) that recruit Th17 cells into the tumour sites, and by the secretion of pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-23 and TGF $\beta$ ), Th17 cells undergo differentiation and expansion, which suppresses further the tumour microenvironment (Su, Ye et al. 2010).

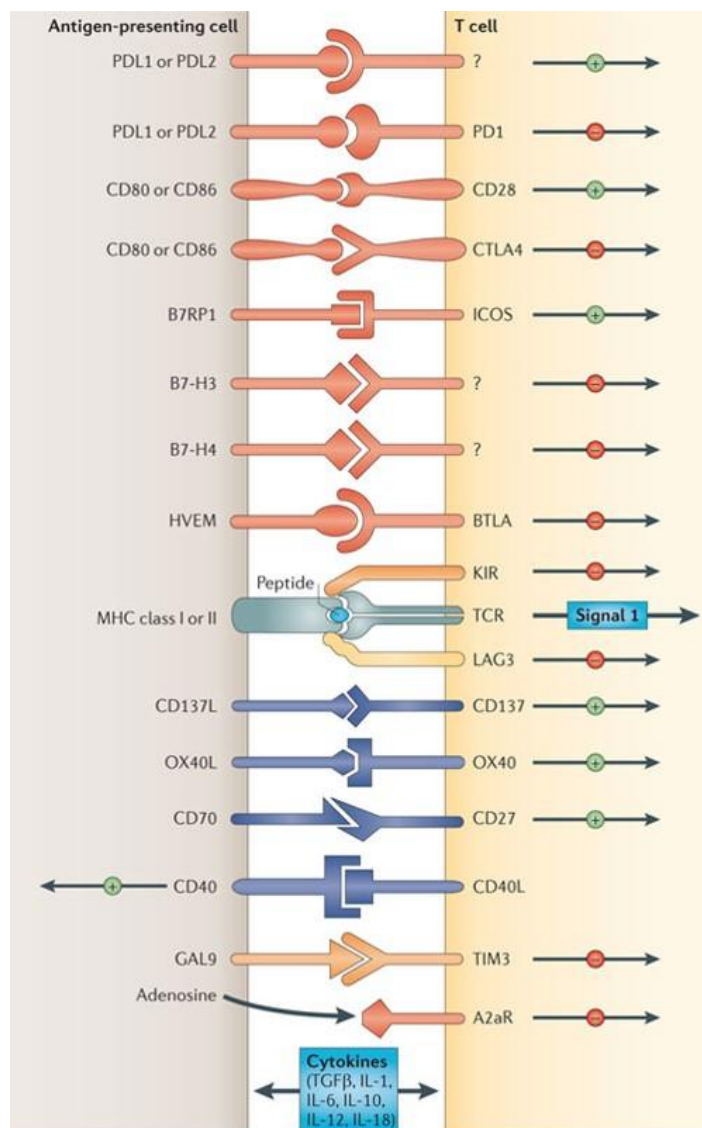
Another factor that was implicated in the immunosuppressive activity is the prostaglandins of the series E (PGE), derived from the seminal plasma. It has been reported that they suppress the lymphocyte and NK cell proliferation and activation. Additionally they are involved in the antibody class switching, as well as in the polarization of Th1 cells to a Th2 pattern (Kelly 1995).

So it is suggested that while the immune system is able to recognise and attack the tumour cells, the immunosuppression in the cancerous microenvironment that is caused by various factors, allows the tumour cells to escape and suppress the immune responses directed against them.

### 1.3.2 Immunological checkpoints

The immunological checkpoints represent a balance that is kept between co-stimulatory and inhibitory signals in the immune system, which under normal conditions are important in maintaining homeostasis and self-tolerance, to prevent autoimmunity and to protect tissues from damage when the immune system responds to a foreign antigen. The interactions between ligands and receptors are often manipulated by tumours, as a mechanism for tumour cell escape from the immune system: T cells are capable of directly recognizing and killing target cells (CTLs) and of orchestrating immune responses from both the innate and adaptive immunity, hence agonists of co-stimulatory receptors or antagonists of inhibitory signals have been the main focus of the design of novel cancer immunotherapeutics. Figure 1.4 shows a variety of receptor-ligand interactions between T cells and APCs that regulate the T cell response to a foreign antigen, adapted by (Pardoll 2012).





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**Figure 1.4: Pairs of co-stimulatory- inhibitory receptors and their corresponding ligands.** The T cell response to a foreign antigen is dependent on the receptor-ligand interactions between T cells and Antigen Presenting Cells (APCs). Figure adapted by (Pardoll 2012).

These immune checkpoints were found to contribute to the immunosuppression in the prostate gland and since then they have become targets of immunotherapy (Pardoll 2012).

CTLA-4 is an inhibitory receptor, expressed on activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, binding to B7-1 (CD80) and B7-2 (CD86) co-stimulatory molecules on APCs; it was found that it is also expressed on Treg cells, thus contributing to immunosuppression (Wing, Yamaguchi et al. 2011). Furthermore, its blockade has emerged as an important immunological adjuvant for DNA vaccines in cancer immunotherapy (Gregor, Wolchok et al. 2004).

PD-1 (Programmed Death-1) is a co-inhibitory receptor (the other receptor is PD-2), also expressed on activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B and NK cells and even monocytes post cytokine stimulation, which limit the immune cells' target lysis. PD-1 binds to the PD-L1 ligand on APCs and their interaction leads to downregulated T cell proliferation due to the inactivation of effector molecules such as Zap70 (zeta chain of T cell receptor associated protein kinase 70) (Agata, Kawasaki et al. 1996). Furthermore it is shown that this interaction promotes the CD4 T cell conversion to Treg cells, thus preventing autoimmunity (Dong, Strome et al. 2002, Blank, Brown et al. 2004). It has been reported that PD-1 enhances tumour cell proliferation and tumour growth after ligand binding, PD-L1, which is over-expressed in cancer cells, stromal cells and TILs (Zou and Chen 2008, Topalian, Drake et al. 2012). PD-1, has been found to be expressed at lower levels in cells derived from PCa patients, unlike the cells from healthy donors, suggesting that the lymphocytes involved in the PCa eradication are inhibited or exhausted (Freeman, Long et al. 2000, Ebelt, Babaryka et al. 2009). Moreover, increased numbers of FoxP3<sup>+</sup>, PD-1<sup>+</sup> and PD-L1<sup>+</sup> lymphocytes were detected in PCa lesions, which suppressed the anti-tumour immunity (Ebelt, Babaryka et al. 2009). Castrate-resistant PCa (CRPC) patients, that were refractory to anti-androgen enzalutamide treatment, showed an increased frequency of PD-L1<sup>+</sup> DCs (Bishop, Sio et al. 2015). One study reported the stromal growth factors CCL2, IL-6 and TGFβ released by stromal myofibroblasts in the PCa microenvironment, induced monocyte differentiation into a suppressive DC phenotype (CD14<sup>+</sup>, PD-L1<sup>+</sup> DCs), which further contributed in the immunosuppression of the cancerous prostate (Spary, Salimu et al. 2014).

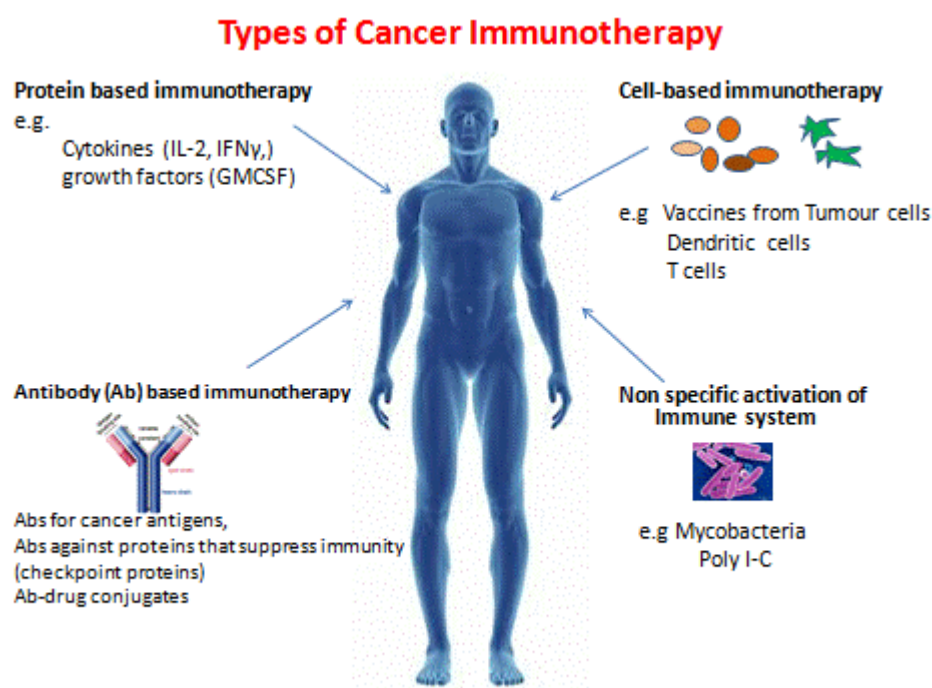
B7-H3 and B7-H4 are type I transmembrane proteins, with unknown receptor to date, that are expressed on activated APCs and have been implicated in immunomodulatory processes in PCa, and enhancing the tumour progression in various cancer types such as gastric, prostate and renal cell carcinoma (Zang, Thompson et al. 2007, Arigami, Uenosono et al. 2010, Qian, Yao et al. 2010). Overexpression of B7-H3 has been associated with increased frequency of TILs, increased tumour growth and progression to the metastasis stage in various cancer types, such as breast, hypopharyngeal squamous cell carcinoma and gastric carcinoma (Wu, Jiang et al. 2006, Arigami, Narita et al. 2010, Katayama, Takahara et al. 2011). *In vitro* experiments with melanoma cell lines showed that silencing B7-H3 inhibited metastasis, by downregulating MMP-2 (matrix metalloproteinase-2), involved in tumour cell migration, invasion and metastasis, with a concomitant increase in TIMP-1 and TIMP-2 (TIMP metalloproteinase inhibitors 1 and 2), involved in MMPs inhibition. It also downregulated STAT-3 phosphorylation levels, involved in metastasis and promoting cell signalling, and finally downregulated IL-8 which promotes melanoma metastasis (Tekle, Nygren et al. 2012). In regards to PCa, molecules of the B7 family were found to be present on 93-99% of PCa cells correlating with a poor disease prognosis in patients (Roth, Sheinin et al. 2007, Zang and Allison 2007). B7-H3 was additionally associated with higher Gleason score, higher Ki67 proliferation marker and larger tumour volume and found to be expressed in bone metastasis and hormone-resistant PCa specimens (Roth, Sheinin et al. 2007, Chavin, Sheinin et al. 2009, Liu, Vlatkovic et al. 2012). Interestingly, a recent *in vivo* study in TRAMP mice directly associated lack of B7-H3 and not of B7-H4 with an increased Treg cell accumulation in the PCa microenvironment, suggesting that the presence of B7-H3 has an inhibitory mechanism towards the FoxP3<sup>+</sup> Treg expression (Kreymborg, Haak et al. 2015).

TIM3 (T cell immunoglobulin domain and mucin domain-containing molecule 3), is another immune checkpoint receptor that has recently been reported to cause immune suppression in cancer. It is expressed exclusively on Th1 CD4<sup>+</sup> T cells and upon binding to its ligand, Galectin-9, it is thought to inhibit Th1 and Th17 responses (Sakuishi, Jayaraman et al. 2011). In preclinical models of solid and haematological malignancies, a suppressive population of CD8<sup>+</sup> T cells that co-expressed TIM3 and PD-1, exhibited defects in proliferation and cytokine production, enhancing tumour growth (Sakuishi, Apetoh et al. 2010). In PCa specimens, TIM3 has been correlated with higher Gleason score, poor prognosis of disease

and disease progression (Piao, Jin et al. 2014). TIM3 blockade on PCa tumour models (TRAMP-C1 prostate carcinoma) resulted in tumour regression, however blockade of both TIM3 and PD-1 resulted in an even greater tumour regression, suggesting that utilizing combinatorial blockade of immune checkpoint inhibitors could enhance the anti-tumour immunity.

## 1.4 Cancer immunotherapeutic strategies

The manipulation of the host and tumour cell interactions in order to stimulate the immune cells to recognise and target the cancer cells, is the primary aim of the current immunotherapeutic approaches that are used in cancer treatment. Various immunotherapeutic modalities exist for the treatment of several cancers; these include protein-based immunotherapies, antibody-based, cell-based and through the non-specific activation of the immune system (Figure 1.5).



**Figure 1.5:** Current immunotherapeutic approaches that are used for the treatment of various cancer types.

### 1.4.1 The use of cytokines in protein-based immunotherapies

Non-cell based immunotherapies include the use of growth factors (e.g. GM-CSF), or the use of cytokines; these bind to cell surface receptors, stimulating the immune cell growth and activation. Cytokine treatment aims to stimulate the immune system cells to trigger a response, either by increasing the immune cell proliferation with a concomitant activated cytotoxicity towards the cancer cells, or by cytokine production which increases the immune reactivity (Smyth, Cretney et al. 2004). Notable examples of cytokines that are used in cancer treatment, are IL-2 and IFN $\alpha$ , which were approved by the Food and Drug Administration (FDA) in 1992 and 2011 respectively, for their use in metastatic melanoma treatment, and in 1998 and 2009 respectively, for their use in renal cell carcinoma (RCC), based on their pleiotropic effects on immune cells (Belardelli, Ferrantini et al. 2002, Gaffen and Liu 2004).

IL-2 has myriads biological effects on immune cells, including the expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, augmenting their cytokine production and hence their cytolytic activity. Additionally, it stimulates tumour-infiltrating lymphocytes to target and attack the cancer cells (Gaffen and Liu 2004).

270 patients with metastatic melanoma, treated with IL-2 and enrolled into clinical trials between 1985 and 1993, were reviewed; 17% of patients showed complete response whereas 10% showed partial response. It was concluded that IL-2 should be considered as a therapeutic option for metastatic melanoma, as no responder showed further disease progression (Atkins, Kunkel et al. 2000).

In the case of RCC, 259 patients who were treated with high doses of IL-2, were reviewed for the period between 1986 and 2006; the complete responders were 23%, whereas the partial responders were 30%. Only 4 complete responders developed disease recurrence, but all the partial responders experienced disease recurrence. IL-2 caused toxicities in many patients, but only 2 of them died of these. Overall, it was concluded that high dose of IL-2 produced tumour regression and despite the toxicities associated with it, it should still be considered as a treatment for patients with RCC (Belldegrun, Klatte et al. 2008, Klapper, Downey et al. 2008). In a separate study, it was shown that localized injection of IL-2 into a skin lesion can promote systemic activation of anti-tumour immunity to clear distant tumours (Johnson, Lum et al. 2008). Furthermore, Schwartzentruher *et al* (2011) showed that the administration of a synthetic peptide vaccine together with IL-2 prolonged a progression-free and overall

survival of melanoma patients, unlike IL-2 monotherapy (Schwartzentruber, Lawson et al. 2011).

IFN $\alpha$  also enhances the cytolytic activity of lymphocytes as well as increasing the macrophage phagocytic activity and is considered as adjuvant therapy in patients with RCC and melanoma (Coppin, Porzsolt et al. 2005, Mocellin, Pasquali et al. 2010). Furthermore, combination of IL-2 and IFN $\alpha$  treatments increased the overall survival of RCC patients with lung metastases (Negrier, Escudier et al. 1998).

IL-12 is a cytokine produced by APCs in response to an antigenic stimulus, promoting CD4<sup>+</sup> T cell polarization to Th1, promotes anti-tumour responses and inhibits tumour derived Treg cells (Kilinc, Aulakh et al. 2006, Zhao, Zhao et al. 2012). It is another example of interleukins that showed some promising results in cancer treatment, as in the case of T cell lymphoma (Rook, Wood et al. 1999), non-Hodgkin's B cell lymphoma (Younes, Pro et al. 2004) and AIDS-associated Kaposi sarcoma (Little, Pluda et al. 2006).

IL-15 is a pro-inflammatory cytokine that plays an important role in autoimmunity and inflammation. Its mRNA is expressed by a variety of tissues and cell types, including fibroblasts, keratinocytes, nerve cells, monocytes and macrophages (Anderson, Kumaki et al. 1995). There is little evidence known indicating that IL-15 plays a direct role in the development of solid cancers (Lissoni, Rovelli et al. 1998); rather it may offer a protective role in the immunosurveillance during a tumour formation. There are several current ongoing clinical trials involving IL-15 for the treatment of various cancer types such as RCC, melanoma and acute myeloid leukemia (AML).

IL-21 is a pleiotropic cytokine that bridges the innate and adaptive immunity; activated CD4<sup>+</sup> T cells produce it, whereas upon cell activation, it expands the CD8<sup>+</sup> T cells and aids their cytotoxic activities (Hinrichs, Spolski et al. 2008). Several preclinical studies have presented the anti-tumour effects of IL-21 in various *in vivo* tumour models, such as in the case of B16 melanoma and MethA fibrosarcoma, where IL-21 was used in a vaccine platform that provided 50% protection before, and 70% cure after tumour challenge of non-immunogenic tumours (Ma, Whitters et al. 2003). Furthermore, IL-21 was shown to promote immune protection to mice in a colon carcinoma model (Ugai, Shimozato et al. 2003).

It is crucial to point out that these cytokines are used systemically and they can be extremely toxic, so they are only suitable for patients that are refractory to any other treatment (Fehniger, Cooper et al. 2002). Their localization to the cancerous lesion would

both increase their efficacy and lower their toxicity. Also when considering such agents for immunotherapies – it is vital to consider their actions at the site of the lesion where the immune system is most compromised. Many cytokines will have no effect when injected into this immunosuppressive environment based on the preliminary *in vitro* data described on chapter 3.

### 1.4.2 Antibody-based immunotherapies

Recent studies in cancer immunotherapy have focused on the concept of immune checkpoints and on the development of antibodies against immunological checkpoint proteins that are used either alone or in combination with other drugs.

The first fully human FDA- approved monoclonal antibody was Ipilimumab, targeted against CTLA-4, for the treatment of metastatic melanoma. Long term follow-up of patients with metastatic melanoma that were treated with ipilimumab, showed an overall survival in 19-36% of patients, with an up to 4 years life extension and manageable immune related adverse events (Hodi, O'Day et al. 2010, Prieto, Yang et al. 2012, Lebbe, Weber et al. 2014). Based on these results, ipilimumab is currently in trials for the treatment of non-small cell lung carcinoma, metastatic RCC and ovarian cancer (Tse, Collins et al. 2014, Garon 2015, Parekh and Rini 2015).

In PCa in a phase I clinical trial involving CRPC patients, combination of ipilimumab with GM-CSF induced an expansion of circulating CD25<sup>+</sup> CD69<sup>+</sup> CD8<sup>+</sup> T cells as well as a decline in the PSA levels in 50% of patients that received the highest doses of anti-CTLA-4 antibody. Furthermore, one patient showed a partial response in visceral metastasis (Fong, Kwek et al. 2009).

Two phase III trials have been initiated; the first was investigating the effects of ipilimumab versus placebo in patients with metastatic CRPC that had progressed after docetaxel treatment, and the initial findings showed no significant difference between the two groups in terms of the overall survival benefit. However, further subgroup analyses showed that ipilimumab offered a greater survival benefit (~6.9 months increased survival) to patients with specific baseline characteristics, such as alkaline phosphatase <1.5 times the upper limit of normal and haemoglobin >11.0g/dL (Kwon, Drake et al. 2014). The second clinical trial (NCT02279862) is investigating the safety and efficacy of different doses of ipilimumab in

patients with metastatic CRPC, with no prior chemotherapeutic agent administration. It is currently ongoing, hence there are no results available yet.

Tremelimumab is another fully human monoclonal antibody against CTLA-4, which was investigated in phase I trials in PSA-recurrent PCa patients after surgery and/or radiation therapy, however no significant changes were observed in PSA doubling time shortly after completing the treatment (McNeel, Smith et al. 2012).

Two recently FDA-approved monoclonal antibodies targeted against PD-1 for the treatment of metastatic melanoma are Nivolumab and Pembrolizumab; the first is given in patients who are already treated with ipilimumab or serine/threonine protein kinase B-Raf (BRAF) inhibitor (Robert, Long et al. 2015, Weber, D'Angelo et al. 2015), whereas pembrolizumab is given in patients without prior ipilimumab treatment (Sullivan and Flaherty 2015). In 2015, the FDA also approved nivolumab for the treatment of refractory squamous non-small cell lung carcinoma (Topalian, Hodi et al. 2012).

No study to date has proven that nivolumab monotherapy is efficacious in PCa patients, however there are two currently ongoing clinical trials; the first one (NCT02601014) is testing combination therapy of ipilimumab with nivolumab in metastatic hormone-resistant PCa patients and the second (NCT02499835) is studying the combination of a DNA vaccine therapy with pembrolizumab in the treatment of patients with hormone-resistant PCa.

### **1.4.3 Non-specific immunotherapies**

Non-specific immunotherapies focus on widespread changes that will lead to the immune system activation, without the need of inducing responses to specific antigens. Such therapies include the use of mycobacteria, immune stimulatory agents such as CpG oligonucleotides, or enzyme inhibitors such as of indolamine-2,3-dioxygenase (IDO).

CpG oligonucleotides activate innate effector cells and APCs via toll-like receptor signalling and additionally they are thought to suppress the activity of myeloid-derived suppressor cells (Shirota, Shirota et al. 2012).

IDO has various immunosuppressive effects in the tumour microenvironment as it is over-expressed in the tumour-infiltrating immune lymphocytes. It induces Treg cell expansion in the tumour microenvironment and it inhibits the NK and T cell proliferation as well as their activation (Munn and Mellor 2007). Hence it is crucial to inhibit the actions of such an agent



and indeed there have been several IDO inhibitors developed and tested, such as 1-Methyl-D-tryptophan. The latter has been tested in synergy with chemotherapeutic drugs (e.g. Doxorubicin, cyclophosphamide) *in vivo* in RAG-1 deficient hosts (lacking T and B cells), inducing antitumor immune responses (Hou, Muller et al. 2007).

#### 1.4.4 Cell-based immunotherapies and vaccines

Cell based immunotherapies have produced less beneficial survival outcomes in comparison to passive immunotherapies (e.g. Antibody-based) overall, however, in the past few years that trend has changed. That is due to our better understanding of how the tumour cells evade the immune system. Cancer vaccines have become an attractive strategy to induce immune effector cell activation and subsequent tumour cell lysis, by utilizing tumour-associated antigens and suitable adjuvants to induce an immune response. Cancer vaccines range from DC-based, DNA-based and recombinant protein-based to whole tumour cell vaccines.

The PCa has been identified as an ideal malignancy for cancer vaccine targeting, due to the unique antigens that are expressed both by normal and cancerous cells, such as PSA and prostatic acid phosphatase (PAP).

Sipuleucel-T (Provenge™) was the first autologous DC-based vaccine approved by the FDA in 2010 for the treatment of metastatic CRPC and to date it is the only approved immunotherapeutic treatment for PCa (Sims 2012). It exploits the patient's immune cells to target the cancer: peripheral blood mononuclear cells are isolated from the patient through leukapheresis and are then cocultured for 36 hours with a fusion protein combining PAP (indicative of cancer progression) and GM-CSF (added to stimulate APCs and obtain more mature DCs). They are then re-infused into the patient, and this product contains a very high number of DCs ( $5 \times 10^7$  cells), as well as a variable number of NK, T and B cells (Small, Schellhammer et al. 2006). This procedure is repeated three times every two weeks and it has shown to improve survival by 4.1 months, however it is a very expensive vaccine, costing around £80,000 per patient and only indicated for stage IV metastatic disease, when the patient's immune system is already weakened. Hence, although Sipuleucel-T can activate cells such as natural killer (NK) cells and cytotoxic T cells to infiltrate the prostate, the tumour

microenvironment renders these cells anergic and/or suppressive, therefore tolerance *in situ* is not reversed.

In a retrospective analysis of the data from the IMPACT (Immunotherapy for Prostate Adenocarcinoma Treatment) trial study, where Sipuleucel-T was compared to placebo in a phase III clinical trial, the patients with lower PSA levels at baseline had an increased survival benefit of 13 months compared to placebo, evidencing that immunosuppression leads to a higher tumour burden (Schellhammer, Chodak et al. 2013).

Sipuleucel-T is currently tested in clinical trials in combination with other drugs like ipilimumab (NCT01832870), the radiotherapeutic drug Radium-223 (NCT02463799) and the hormone therapeutic drugs Abiraterone (NCT01487863) and Enzalutamide (NCT01981122).

GVAX (granulocyte-macrophage colony-stimulating factor tumour cell vaccine) is an example of a whole cancer-cell based vaccine that was tested for the treatment of hormone-refractory PCA patients. It is derived from two allogeneic PCa cell lines, LNCaP (androgen dependent cell line, derived from lymph node metastasis) and PC-3 (androgen independent cell line, derived from bone metastasis), that are transfected with GM-CSF and irradiated for safety purposes. In phase I/II trials, chemotherapy-naïve with hormone-refractory PCa patients received GVAX and the results showed a safe toxicity profile as well as an increased overall survival of about 35 months in the high-dose group of patients. Based on these results two phase III trials started: VITAL-1 (Vaccine Immunotherapy with Allogeneic Prostate Cancer Cell Lines-1), which recruited asymptomatic patients to receive GVAX or docetaxel with prednisone and VITAL-2, which recruited symptomatic patients to receive combination of GVAX with docetaxel or docetaxel alone. VITAL-2 trial was terminated early due to a high mortality rate in the vaccine arm and VITAL-1 was also terminated due to an unplanned futility analysis that indicated that the trial had less than 30% chances of meeting its primary endpoint of an improvement in survival (Higano, Corman et al. 2008).

Nevertheless, GVAX is currently tested in two different trials for the treatment of colorectal (NCT01952730) and of pancreatic cancer (NCT02004262), either alone or in combination with other agents.

## 1.5 Conclusions

The PCa microenvironment is very immunosuppressive and poorly immunogenic since all the infiltrating lymphocytes become anergic or suppressive once they enter the cancerous prostate. That is due to the presence of various suppressive cell populations or growth factors as mentioned earlier. Currently the only immunotherapeutic treatment that is approved by the FDA for PCa is Sipuleucel-T, with minimal results though, extending the lifespan by 4.1 months, and it is considered only at the metastatic stage of PCa, once the patient's immune system is already greatly weakened.

PCa is suitable for immunotherapy as it is a relatively slow growing cancer and immune responses take longer to mount a response compared to chemotherapy. It is critical though, to consider non-cell based immunotherapies for the treatment of PCa, as they have been previously used on patients with successful outcomes, as in the case of IL-2 that is FDA approved for use in melanoma and RCC. However, such cytokines are used systemically and hence can be extremely toxic, so they are only suitable for patients that are refractory to any other treatment. It has been further established that localized injection of IL-2 into a skin lesion can promote systemic activation of anti-tumour immunity to clear distant tumours (Johnson, Lum et al. 2008). Although when injected systemically, these proteins are very toxic, their localisation to the cancerous lesion would both increase their efficacy and lower their toxicity. Also when considering such agents for immunotherapies – it is vital to consider their actions at the site of the lesion where the immune system is most compromised. Many cytokines will have no effect when injected into this immunosuppressive environment.

Lastly, it has been widely observed from several types of immunotherapies for various cancers that monotherapies are less efficacious compared to combinatorial treatments, so that should also be considered when developing a novel immunotherapeutic treatment for cancer.

## 1.6 Aims and Hypotheses

Preliminary data to this project from our laboratory (see Chapter 3), has shown that in an *in vitro* allogeneic coculture model of non-adherent peripheral blood mononuclear cells (PBMCs) with PCa cell lines (originating both from metastatic and non-metastatic cancer), that mimics the PCa microenvironment, IL-15, among a panel of Th1 therapeutic cytokines, was found to be the most potent activator of NK, NKT and CD8 T cells, aiding their cytotoxic activity towards the PCa cell lines (Elhage, Galustian et al. 2012). Based on this preliminary work and on the previous highlighted evidence, a number of hypotheses can be put forward, that were investigated in order to better understand how to harness immunotherapeutic agents such as IL-15 to treat PCa.

These hypotheses are that:

1. PCa can progress by tolerance to, and evasion of tumour cells from the immune-system,
2. Immunomodulatory targets in the PCa microenvironment expressed on either cells or in soluble form, are critical in this PCa progression.
3. The tumour microenvironment represents the site of greatest immune tolerance and therefore, immune therapies targeting at the tumour microenvironment, rather than introduced systemically will break tolerance *in situ* and allow effective elimination of cancer cells.

The aims of this project are:

1. To create *in vivo* and *in vitro* models of prostate tumour-immune effector cell interaction and tumour cell killing, to enable us to identify, test and optimize novel immunotherapeutics
2. To investigate the mechanisms of action of IL-15 on effector cell mediated killing of PCa cells in these models, focusing particularly on NK and NKT cells.

The NK receptors and their ligands will be particularly studied, to investigate whether IL-15 modulates the expression and shedding of these ligands.

3. To determine the efficacy of novel immunotherapeutics both singly and in combination, on the ability of NK and NKT cells to kill prostate tumour cells.

## CHAPTER 2

### Materials & Methods

## **2.1 Materials and Reagents:**

### **2.1.1 Plasticware**

25cm<sup>2</sup>, 75cm<sup>2</sup>, 175cm<sup>2</sup> tissue culture (TC) treated flasks (Greiner Bio-One Ltd, Stonehouse, UK)

5ml, 10ml, 25ml serological pipettes (Greiner Bio-One Ltd)

15ml, 50ml centrifuge tubes (Greiner Bio-One Ltd)

6, 12, 24, 48, 96 – well plates, tissue culture treated (Greiner Bio-One Ltd)

Filter system 0.22µm pore size, sterile (Corning, Flintshire, UK)

1.5ml microcentrifuge tubes (ThermoFisher Scientific, Paisley, UK)

1.5ml Cryovials (Corning)

10ml Vacutainer™ tubes (Greiner-Bio One)

40µm cell strainers (Greiner-Bio One)

Polysine™ slides Menzel-Gläser (ThermoFisher Scientific)

Cover slips 24\*64mm No 0 (SLS, Nottingham, UK)

G-25 mini columns (GE Healthcare, Buckinghamshire, UK)

3K desalting columns (Amicon, Watford, UK)

Float-A-Lyzer dialysis units (Spectrum labs, California, USA)

### **2.1.2 Molecular Biology Reagents**

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Gillingham, UK)

Ethanol (Sigma-Aldrich)

Acetone (Sigma-Aldrich)

Phytohemmagglutinin (PHA) (ThermoFisher Scientific)

### **2.1.3 Tissue culture reagents**

RPMI-1640 Medium with Sodium Bicarbonate (Sigma-Aldrich)

RPMI-1640 Medium, no phenol red (Life Technologies, Paisley, UK)

Dulbecco's Modified Eagle's Medium (DMEM)- high glucose (Sigma-Aldrich)

Hanks' balanced salt solution (HBSS) (Sigma-Aldrich)  
Gentamicin solution sterile filtered (Sigma-Aldrich)  
Accutase (Sigma-Aldrich)  
Phosphate buffered saline (Severn Biotech Ltd, Worcestershire, UK)  
L-glutamine solution (Sigma-Aldrich)  
Antibiotic-antimycotic solution (Sigma-Aldrich)  
MycoZap prophylactic (Lonza, Berkshire, UK)  
Trypan blue (Sigma-Aldrich)  
Foetal Bovine Serum Certified (Life technologies)  
Bovine insulin (Sigma-Aldrich)  
Dehydroisoandrosterone (Sigma-Aldrich)  
CellTitre 96® Aqueous One Solution Cell Proliferation Reagent (Promega, Southampton, UK)  
Histopaque-1077 Hybri-max (Sigma-Aldrich)  
10x RBC lysis buffer (eBioscience, Altrincham, UK)  
Diamedix™ 24EZ Complement Cells (Fisher Scientific, Paisley, UK)

#### **2.1.4 Antibodies and cytokines**

Human Interleukin-2 (Peprotech, London, UK)  
Human Interleukin -12 (Peprotech)  
Human Interleukin -15 (Peprotech)  
Human Interleukin -21 (Peprotech)  
Human IFN $\gamma$  (Peprotech)  
Murine Interleukin-2 (Peprotech)  
Murine Interleukin -12 (Peprotech)  
Murine Interleukin -15 (Peprotech)  
Murine Interleukin -21 (Peprotech)  
Murine IFN $\gamma$  (Peprotech)  
Recombinant human Interleukin-15 (Genscript, Hong Kong, China)  
Ultra-LEAF™ Purified anti-human CD314 (NKG2D) Antibody [Clone: 1D11] (Biolegend UK Ltd, London, UK)

Ultra-LEAF™ Purified Mouse IgG1, κ Isotype Ctrl Antibody [Clone: MOPC-21] (Biolegend UK Ltd)

Human MICA MAb (Clone 159227) (Bio-technie, Abingdon, UK)

Mouse IgG2B Isotype Control (Clone 20116) (Bio-technie)

Annexin V apoptosis detection kit APC (eBioscience)

Fc block, human or mouse (Miltenyi Biotec, Surrey, UK)

Anti-PTL033, Clone UC10-4F10-11 (BioXcell, New Hampshire, USA)

Anti-PTL035, Clone 10F.9G2 (BioXcell)

Recombinant mouse PTL033/Fc Chimera (SinoBiologicals Inc., Beijing, China)

Recombinant mouse PTL035/Fc Chimera (SinoBiologicals Inc.)

Antibodies used in fluorescence-associated cell sorting (FACS) experiments are listed in Table 2.1.



Species	Antigen	Fluorophore	Clone	Manufacturer
Human	CD3	FITC	HIT3a	SantaCruz Biotechnology, Heidelberg,
Human	CD3	PE	HIT3a	Biolegend
Human	CD3	APC	HIT3a	Biolegend
Human	CD8	PerCP	BW135/80	Miltenyi Biotec
Human	CD56	PE	MEM-188	Biolegend
Human	CD56	APC	MEM-188	Biolegend
Human	FoxP3	PE	206D	Biolegend
Human	CD107a	APC	H4A3	Biolegend
Human	Perforin	FITC	dG9	Biolegend
Human	CD158a/h	FITC	HP-MA4	Biolegend
Human	CD158b	FITC	DX27	Biolegend
Human	CD158e1	FITC	DX9	Biolegend
Human	CD226	FITC	11A8	Biolegend
Human	CD314	FITC	1D11	Biolegend
Human	CD335	FITC	9E2	Biolegend
Human	CD336	Alexa Fluor 488	3.43	Biolegend
Human	NKp30	FITC	P30-15	Biolegend
Human	MICA/B	FITC	6D4	BioRad AbD Serotec, Paisley, UK
Human	MICA	FITC	4	Biorbyt, Cambridge, UK
Human	MICA	PE	159227	Biolegend
Human	ULBP1	FITC	polyclonal	Biorbyt
Human	Nectin-2	FITC	polyclonal	Biorbyt
Human	HLA-A,B,C	FITC	W6/32	Biolegend
Human	HLA Class I	FITC	Tu149	Life Technologies
Human	HLA-I Bw4	FITC	0.L.6	US Biologicals, California, USA
Human	HLA-G	FITC	4H84	SantaCruz Biotechnology
Human	IL-15	PE	IC2471D	Bio-Techne
Human	PTL035	PE	29E.2A3	eBioscience
Human	PTL033	APC	L3D10	Biolegend
Murine	NK 1.1	Alexa Fluor 647	PK136	Biolegend
Murine	CD3	FITC	17A2	Biolegend
Murine	CD4	FITC	GK1.5	eBioscience
Murine	CD8	PerCP/Cy5.5	53-6.7	Biolegend
Murine	CD25	FITC	3C7	Biolegend
Murine	FoxP3	PE	MF-14	Biolegend

**Table 2.1: Antibodies used for FCAS staining**

### 2.1.5 Biochemistry reagents

Phosphate buffered saline x1 sterile (Severn Biotech)

Bovine Serum Albumin (Sigma- Aldrich)

Cell fix 10x (BD Biosciences, Oxford, UK)

TCEP (ThermoFisher Scientific)

12% and 4-12% Bis-Tris NuPage™ gels (Life Technologies)

NuPage™ LDS loading buffer (Life Technologies)

Mark12™ Unstained Standard (ThermoFisher Scientific)

Novex® Sharp Unstained Protein Standard (ThermoFisher Scientific)

SafeStain (GE Healthcare)

SimplyBlue™ SafeStain (ThermoFisher Scientific)

iBlot® Dry Blotting System (Life Technologies)

Caseine (Sigma-Aldrich)

SIGMAFAST™ 3,3'diaminobenzadine tablets (Sigma Aldrich)

AnaTag™ (AnaSpec, Inc, California, USA)

Hydrogen peroxide solution 30% (w/w) in H<sub>2</sub>O (Sigma-Aldrich)

### 2.1.6 Cytotopic modification agent

PTL3146: The tail used for modification of the IL-15 and the two antibodies to immune checkpoint inhibitors was kindly provided by Dr Richard Smith, Director of the Protein Therapeutics Laboratory at the MRC Centre for Transplantation, King's College London. The tail sequence is ( $\alpha,\epsilon$  bis-myristoyl lysine) SSKSPSKDDKKPGD(S-2-pyridyldithio)-C-acid.

### 2.1.7 Commercial kits: ELISA, Isolation Kits, Intracellular staining kit, Biochemical assays

Pan T cell Isolation Kit II, mouse (Miltenyi Biotec)

NK Cell Isolation Kit II, mouse (Miltenyi Biotec)

MICA ELISA kit, human (Promocell, Heidelberg, Germany)

Human CD112/ Nectin-2/ PVRL2 ELISA (SinoBiologicals, Inc)  
Human IL-2 ELISA MAX™ Deluxe (Biolegend)  
Cytotfix/Cytoperm Soln Kit (BD Biosciences)  
SilverXpress® Silver staining kit (Life Technologies)  
Pierce™ Coomassie (Bradford) Protein Assay Kit (ThermoFisher Scientific)

### 2.1.8 Buffers

Buffers that were used regularly in this thesis and that are not components of commercial kits, are listed below:

#### **FACS buffer**

1% PBS (OXOID, Hampshire, UK)  
0.1% Sodium Azide (Severn Biotech)  
1% Bovine Serum Albumin (Sigma Aldrich)

#### **Isolation Buffer**

0.5% Bovine Serum Albumin (Sigma Aldrich)  
2mM EDTA (Sigma Aldrich)  
PBS (Severn Biotech)  
pH 7.2, sterile filtered

### 2.1.9 Cell culture media and Serum

**Foetal Bovine Serum (FBS):** FBS was heat inactivated by incubation in a water bath at 57°C for 1 hour and stored in aliquots at -20°C thereafter.

**RPMI complete medium:** RPMI-1640 supplemented with 10% FBS, 10ml/L antibiotic/antimycotic solution, 2mM L-glutamine, 20µg/ml gentamicin, 1x mycozap prophylactic. Sterile-filtered. RPMI phenol-free complete medium was prepared with the same supplements.

**DMEM complete medium:** DMEM- high glucose medium supplemented with 10% FBS, 10ml/L antibiotic/antimycotic solution, 2mM L-glutamine, 20µg/ml gentamicin, 1x mycozap

prophylactic, 5% Nu Serum IV, 10nM dehydroisoandrosterone, 0.005mg/ml bovine insulin. Sterile filtered.

### 2.1.10 Cell lines

**PC-3** (ATCC® CRL-1435™): An epithelial cell line derived from a 62- year- old Caucasian male with a grade IV prostate adenocarcinoma, metastasised to the bone. It's highly tumorigenic androgen- independent cell line that was first established in 1979 (Kaighn, Narayan et al. 1979). The PC-3 cell line was obtained from ATCC.

**LNCaP** (ATCC® CRL-1740™): An epithelial cell line derived from the left supraclavicular lymph node of a 50- year- old Caucasian male with a metastatic prostate carcinoma. It is an androgen dependent cell line that was first established in 1980 (Horoszewicz, Leong et al. 1980). The cell line was obtained from ATCC.

**CTLL-2** ATCC® TIM-214™): A cell line that is a clone of cytotoxic T cells derived from a C57BL/6 mouse. They were first established in 1977 (Gillis and Smith 1977).

**TRAMP-C1** (ATCC® CRL-2740™) **and – C2** (ATCC® CRL-2731™) : Transgenic Adenocarcinoma of the Mouse Prostate cell lines 1 and 2 were derived from a 32-week-old C57BL/6 mouse with a primary prostate tumour. They are of epithelial origin and were first established in 1996 (Greenberg 1996). Both cell lines are highly tumourigenic when injected in C57BL/6 mice, although TRAMP-C1 is reported to grow faster than TRAMP-C2 *in vitro* and *in vivo* (Foster, Gingrich et al. 1997). Both cell lines were obtained from ATCC.

**EL4** (ATCC® TIM-39™): A cell line resembling T lymphocytes that was isolated from a lymphoma in a C57BL/6 mouse (Gorer 1950, Old, Boyse et al. 1965). This cell line was a kind donation by Dr. Giorgia Fanelli from the Immunoregulation Laboratory at the MRC Centre for Transplantation, King's College London.

**Jurkat** (ATCC® TIB-152™): A T-lymphocyte-like cell line that was isolated from the peripheral blood cells of a 14-year old boy with T cell leukaemia (Gillis and Smith 1977). The cell line was also a kind donation by Dr. Giorgia Fanelli.

### 2.1.11 *In vivo* experiments

#### Animals and apparatus

Inbred male C57BL/6 Jax strain mice (catalog number 000664), aged 6-8 weeks, were obtained from The Jackson Laboratory.

Digital Vernier Caliper (Harvard apparatus)

#### Buffers

##### Fc block:

10% FBS (Life technologies)

1% Bovine Serum Albumin (Sigma Aldrich)

1x Blocking buffer (Abcam)

#### Immunohistochemistry/H&E reagents and antibodies

Streptavidin/Biotin Blocking kit (Vector Laboratories Inc.)

Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories Inc.)

Vectashield mounting medium for fluorescence Hard Set (Vector Laboratories Inc.)

Haematoxylin (RA Lamb- ThermoFisher Scientific)

Eosin (RA Lamb- ThermoFisher Scientific)

Xylene (Sigma Aldrich)

Optimal cutting temperature compound (OCT) (Fisher Scientific)

DPX (Diputyl Phthalate containing Xylene) (VWR)

CD3 Armenian hamster monoclonal antibody (Abcam)

CD4 goat polyclonal antibody (SantaCruz Biotechnology)

CD8-a rabbit polyclonal antibody (SantaCruz Antibody)

IL2R $\alpha$  rabbit polyclonal antibody (SantaCruz Biotechnology)

FoxP3 anti-mouse/rat biotin (eBioscience)

NK1.1/CD161 rabbit polyclonal antibody (Bioss Antibodies)

AMCA donkey anti-goat IgG (Stratech Scientific Ltd)

Rhodamine (TRITC) donkey anti-rabbit IgG (Stratech Scientific Ltd)

Alexa Fluor 568 goat anti-hamster IgG (ThermoFisher Scientific)

FITC donkey anti-rabbit IgG (Stratech Scientific Ltd)

Alexa Fluor 488 donkey anti-rat IgG (Strattech Scientific Ltd)

Streptavidin FITC (BD Biosciences)

## **2.2 Methods:**

### **2.2.1 Cell culture**

#### **2.2.1.1 Culture of adherent and suspension cell lines**

All adherent cell lines were grown in 175cm<sup>2</sup> TC flasks, at 37°C in a 5% CO<sub>2</sub> humidified incubator, until 70-80% confluency was reached (every 4-5 days). PC-3 and LNCaP cells were grown in RPMI complete medium, whereas TrampC cells in DMEM complete medium. Their passage included removal of the culture medium and addition of 7ml accutase (detachment reagent) for 10-15min. Cells were then washed in HBSS, resuspended in 5ml of culture medium, counted and seeded at 1.5\*10<sup>6</sup>/ml cell density in 30ml of complete medium.

The CTLL-2 cell line was grown in suspension in 75cm<sup>2</sup> TC flasks, at 37°C in a 5% CO<sub>2</sub> humidified incubator. The cells were maintained at a concentration of 6-9\*10<sup>5</sup> cells/ml by the addition of fresh RPMI complete culture medium once per week and the addition of 10-20IU/ml IL-2 twice per week.

The EL-4 and Jurkat cell lines were grown in suspension in 75cm<sup>2</sup> TC flasks, at 37°C in a 5% CO<sub>2</sub> humidified incubator. The cells were maintained at a concentration of 4-6x10<sup>5</sup> cells/ml by the addition of fresh RPMI complete culture medium three times per week.

#### **2.2.1.2 Cryopreservation and thawing of cells**

For long term storage cells were stored in liquid nitrogen or in the -80°C freezer. For freezing the cells were resuspended in 90% FBS + 10% DMSO freezing medium at concentrations of 2-4x10<sup>6</sup> cells/ml and stored in cryovials.

The vials were thawed in a water bath at 37°C and transferred in RPMI complete medium, centrifuged at 400g for 5 min to remove the excess freezing medium; they were then resuspended in RPMI complete medium and transferred to TC flasks.

### **2.2.1.3 Peripheral Blood Mononuclear Cell (PBMC) Isolation**

PBMCs from healthy donors were isolated by Ficoll- Histopaque density gradient centrifugation, following the protocol described by Malkovsky and Sondel (1987) (Malkovsky and Sondel 1987). Briefly, blood samples from healthy donors were diluted 1:1 with HBSS and layered on top of Histopaque, at a 1:2 Histopaque to blood ratio. The samples were then centrifuged at 1600 RPM, with no deceleration, at room temperature for 30 min. PBMCs were then extracted from the buffy coat, washed with HBSS, resuspended in 10ml of red blood cell lysis buffer and incubated at 37°C in a 5% CO<sub>2</sub> humidified incubator for 10 min. Washing with HBSS proceeded and the cells were then resuspended in RPMI complete medium and counted. Cells were resuspended at a concentration of  $3 \times 10^6$  cells/ml in a 175cm<sup>2</sup> TC flask and incubated for a minimum of 3 hours or overnight. This step is called plastic- adherence step; the non-adherent PBMCs contain the fraction of cells (e.g. T, B cells) that was used for further experiments.

### **2.2.1.4 Murine splenocyte isolation and subsequent NK and T cell purification**

Inbred male C57BL/6 JAX strain mice, aged 6-8 weeks, were obtained from the Jackson laboratory. The spleen was extracted from a mouse, kept in sterile filtered PBS and was processed within minutes of removal from the mouse, to minimise the apoptotic splenocyte number.

The spleen was placed on a 40µm cell strainer placed over a 50ml centrifuge tube and pushed through the strainer with the plunger of a syringe, while keeping both the spleen and the membrane of the strainer wet with PBS. The cells that passed through the strainer were washed with HBSS, resuspended in 5ml of red blood cell lysis buffer and incubated at 37°C in a 5% CO<sub>2</sub> humidified incubator for 5 min. They were then washed with HBSS and resuspended in RPMI complete medium. The cells were counted and subsequent NK or T cell purification proceeded.

Murine splenic T lymphocytes were isolated using a Pan T cell isolation kit (Felio, Nguyen et al. 2009). The NK cells from the splenic lymphocytes were enriched using an NK cell isolation kit (40% purity) (Meinhardt, Kroeger et al. 2012). The manufacturer's protocol was followed for all cell purifications.

## 2.2.2 Creation of *in vitro* allogeneic and syngeneic coculture models of immune effector cell – target cell interaction

### 2.2.2.1 Allogeneic coculture model

The non-adherent fraction of PBMCs was cocultured in RPMI phenol-free complete medium, in the presence or absence of human PCa cell lines (PC-3 or LNCaP), at an effector to target (E:T) ratio that ranged from 0.5:1-20:1, in the presence or absence (control) of human recombinant cytokines in a 24-well plate for 7 days. The cytokines tested were: IL-2 (1 or 10ng/ml), IFN $\gamma$  (2.5ng/ml), IL-12 (2.5ng/ml), IL-15 (2.5ng/ml) and IL-21 (2.5ng/ml). The concentration of target cells was  $2 \times 10^5$  cells/ml and that of the immune effector cells was  $8 \times 10^5$  cells/ml.

This allogeneic coculture model was also used for NKG2D and MICA blocking, where 10 $\mu$ g/ml of aNKG2D blocking antibody or 5  $\mu$ g/ml of aMICA were added to the cocultured cells. Their corresponding isotypes (IgG1 or IgG2b) were used as controls and added on separate wells of cocultured cells at the same concentrations.

### 2.2.2.2 Syngeneic coculture model

NK enriched or T enriched cells from splenocytes of a C57BL/6 mouse, were cocultured in RPMI phenol-free complete medium in the presence or absence of a murine PCa cell lines, called TRAMP-C1, at an E:T ratio of 4:1 – also optimised as in the allogeneic model (results shown in chapter 3)-, in the presence or absence (served as control) of murine recombinant cytokines in a 24-well plate for 9 days. The cytokines tested were: murine IL-2 (1 or 10ng/ml), IFN $\gamma$  (2.5ng/ml), IL-12 (2.5ng/ml), IL-15 (2.5ng/ml) and IL-21 (2.5ng/ml). The concentration of target cells was  $1 \times 10^5$  cells/ml and that of the immune effector cells was  $4 \times 10^5$  cells/ml.



## **2.2.3 Cellular assays**

### **2.2.3.1 Cell surface staining**

Cells were transferred to FACS tubes and washed twice with 1ml FACS buffer and centrifuged. Samples were then Fc blocked for 15 min, and incubated with antibodies at 4°C in the dark for 45 min. Samples were then washed, centrifuged and resuspended in 300µl cell fix. Cells were acquired with a FACS Calibur flow cytometer (BD Biosciences) and analysed using FlowJo software.

### **2.2.3.2 Intracellular staining**

Intracellular staining was performed for the human and murine FoxP3 and perforin antibodies using the Cytofix/Cytoperm, Fixation and Permeabilization kit from BD Biosciences. The protocol was followed as per manufacturer's instructions; briefly, following cell surface staining, the cells were permeabilized using the fix/perm solution at room temperature for 20 min, washed with the perm/wash buffer and centrifuged. The antibodies were added to the samples which were then incubated in the dark at room temperature for 30 min. The cells were then washed with the same perm/wash buffer, centrifuged and resuspended in 300µl cell fix. They were acquired using a FACS Calibur flow cytometer (BD Biosciences) (Cell quest software) and analysed using FlowJo software.

### **2.2.3.3 Annexin V/PI staining for the detection of apoptotic/necrotic PCa cells**

Induction of apoptosis and necrosis were investigated using the Annexin V apoptosis detection kit by eBioscience, which contained annexin V conjugated to APC and propidium iodide (PI) conjugated to PE/PerCP.

Briefly, the cells from each well of the 24-well plate were added to FACS tubes and the wells were washed with 500µl PBS. 200µl of accutase were added to each well, so that all the PCa cells that were attached at the bottom of the plate, detach. The cells were then washed once with PBS, centrifuged for 5 min, washed with 1x annexin binding buffer (1x ABB), centrifuged for 5 min and resuspended in 100µl 1x ABB. 5µl of Annexin V APC were added to each

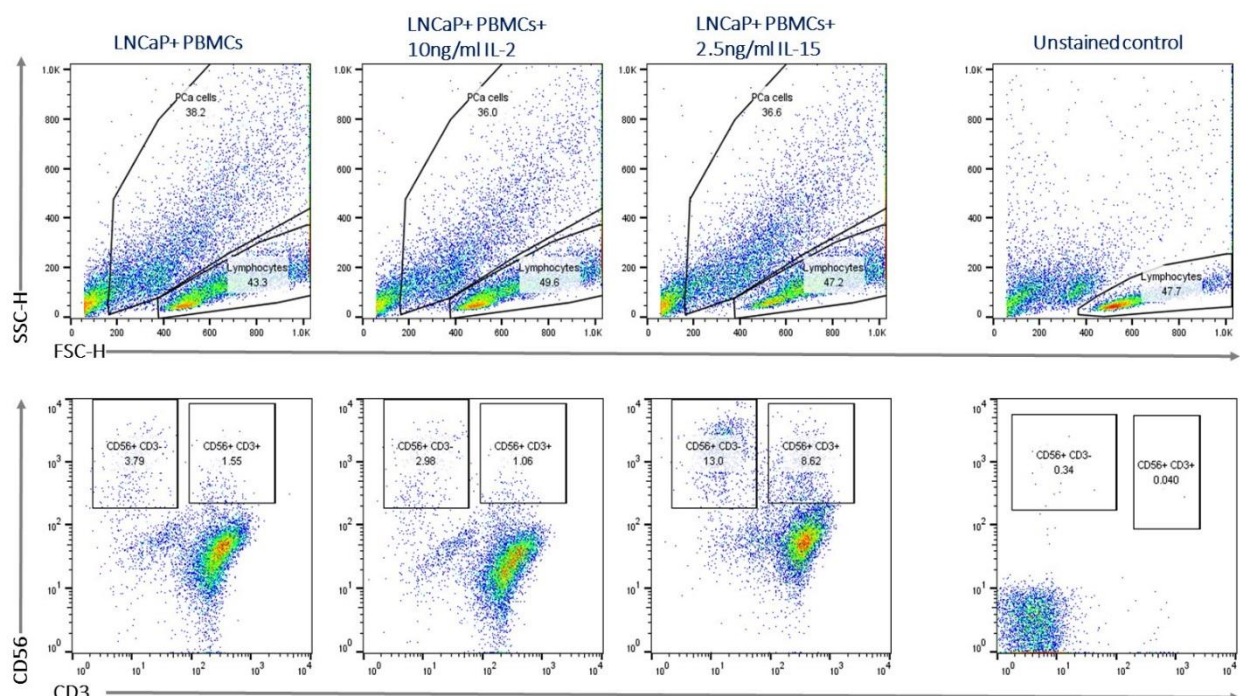
sample, which were then incubated for 15min in the dark. The cells were washed again with 1x ABB, centrifuged for 5 min and resuspended in 200 $\mu$ l 1x ABB. 5 $\mu$ l of PI were added to each sample and they were acquired using a FACS Calibur machine.

In order to observe the maximum cell death that PCa cells could achieve and hence have a positive control for the experiment, 600 $\mu$ M of H<sub>2</sub>O<sub>2</sub> were added to PCa cells in the 24 well plate and incubated for 24 hours. The next day the PCa cells with H<sub>2</sub>O<sub>2</sub> were tested for apoptosis and necrosis.

### 2.2.3.4 Gating strategy and analysis for NK and NKT cells

A representative surface staining gating strategy is shown in figure 6. Non-adherent PBMCs were cocultured with LNCaP cells under different conditions for 7 days and the cells were stained for CD56 and CD3. The gating of viable cells using forward and side scatter is shown on the top panels, where both the location of LNCaP cells is seen as well as the location of the lymphocytes. The bottom panels show the NK and NKT cells from the lymphocytes' gate, as observed by  $CD56^+ CD3^-$  and  $CD56^+ CD3^+$  staining respectively.

Similar approach was adopted on the analysis of other phenotypic markers.



**Figure 2.1: Gating strategy for lymphocytes, NK and NKT cells.** A representative cell surface staining experiment is shown, following coculture of non-adherent PBMCs with LNCaP cells, either without cytokines, or with IL-2 or IL-15 for 7 days. The viable lymphocytes and LNCaP cells on the top panels are gated accordingly. The bottom panels have as a parental population the lymphocytes, from which the NK ( $CD56^+ CD3^-$ ) and NKT ( $CD56^+ CD3^+$ ) cells are gated.

### 2.2.3.5 Viability/proliferation assay to confirm the activity of IL-15

Cell proliferation and viability were measured using the Celltitre96® Aqueous One solution cell proliferation assay (MTS) by Promega. This is a colourimetric assay utilizing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) which when cultured with metabolically active cells result in the generation of a formazan product, which absorbs light at a wavelength of 490nm. The resulting absorbance is directly proportional to the number of viable cells in culture.

The CTLL-2 cells were added to a 96-well plate at a concentration of  $5 \times 10^5$  cells/ml. The cells were incubated for 72 hours in the presence or absence of cytokines and 20µl of the MTS reagent were added to each well (100µl cell suspension). The plate was incubated in the dark at 37°C in a humidified, 5% CO<sub>2</sub> environment for 4 hours. 25µl of 10% SDS were added to each well to stop the reaction. The plate was read at 450nm using a 96-well plate reader (Molecular Devices, UK).

### 2.2.3.6 Jurkat cell- based assay for the detection of tailed PTL033 activity (performed by Dr. Dorota Smolarek)

Jurkat cells at a concentration of  $10^5$  cells per 100µl were added on a 96-well plate and activated using 2.5µg/ml of PHA and 2µg/ml of soluble Fc chimera protein that is responsible for the cell activation. In order to inhibit the cell activation, 2µg/ml of soluble Fc chimera protein to the immune checkpoint inhibitor were added, followed by varying concentrations of the antibody to immune checkpoint inhibitor, tailed or untailed. Cells were then incubated for 4 hours at 37°C in a humidified, 5% CO<sub>2</sub> environment. The plate was centrifuged at 400g for 5mins and the supernatants were collected and analysed for the soluble secretion of IL-2 by ELISA (Biolegend), as per manufacturer instructions.

### **2.2.3.7 EL4 cell- based assay for the verification of tailed PTL035 activity (performed by Dr. Dorota Smolarek)**

EL4 cells at a concentration of  $10^5$  cells per 100 $\mu$ l were incubated with a PE-labelled anti-mouse to the immune checkpoint inhibitor antibody in order to establish binding with the immune checkpoint inhibitor's receptor on their surface, followed by the addition of 50 $\mu$ g/ml Fc to immune checkpoint inhibitor protein, in order to disturb the immune checkpoint inhibitor's receptor binding, followed by the addition of varying concentrations of antibody to the immune checkpoint inhibitor, in order to reconstitute binding of fluorescently labelled antibody to the immune checkpoint inhibitor. The samples were incubated at 4°C in the dark for 45 min. Samples were then washed, centrifuged and resuspended in 300 $\mu$ l PBS. Cells were acquired with a FACS Calibur flow cytometer (BD Biosciences).

## **2.2.4 Biochemical assays**

### **2.2.4.1 MICA and Nectin-2 Enzyme - Linked Immunosorbent Assays (ELISA)**

Supernatants from the allogeneic coculture model were collected and were analysed for the soluble expression of MICA (Allez, Tieng et al. 2007) and of Nectin-2 (Bachelet, Munitz et al. 2006) by ELISAs (Promocell and SINO Biologicals) , as per manufacturer instructions.

### **2.2.4.2 Tailing of IL-15 (performed by Dr. Dorota Smolarek)**

0.5mg/ml of human recombinant IL-15 was incubated (rotating) with TCEP at a final concentration of 300 $\mu$ M, for 45-60min. The mixture was then applied on a G-25 mini column, so that excess TCEP was removed. The tail was added to IL-15 at a 1:3 IL-15 to tail molar ratio and incubated for 60-90 min at room temperature, followed by overnight incubation (rotating) at 4°C. The mixture was then dialysed against 1L of PBS for a day using a dialysis unit (Float-A-Lyzer, Spectra/Por) according to manufacturer's instructions; the PBS was then discarded and fresh PBS was added for an overnight dialysis. The next day the mixture of IL-15 with the tail was analysed on an SDS-PAGE and the concentration was assayed by Coomassie Protein Assay (ThermoFisher Scientific) (Bradford 1976).

#### **2.2.4.3 Tailing of the antibodies to immune checkpoint inhibitors (performed by Dr. Dorota Smolarek)**

The two antibodies of interest were modified with 2-iminothiolane (2-IT), a reagent that is used to incorporate thiol groups into protein by interaction with lysine amino groups (Smith and Cassels 1988).

First the antibodies (1mg/ml), water and PBS were degassed under the vacuum for one hour. 2-IT was dissolved in water at a 100mM concentration. 5µl of 2-IT (final concentration 2mM) was added to the antibody solution and incubated at room temperature for 30min. 8µl of 10mM tail solution (approximately 1:3 antibody to tail molar ratio) were added to the mixture of antibody and 2-IT with vortexing. The reaction mixture was incubated at room temperature for 5min, while monitoring the reaction by measuring its absorbance at A343 with a spectrophotometer. The reaction mixture was then applied on a G-25 mini-column at room temperature and separation of antibody from excess of 2-IT and tail was done using a spin protocol method. The antibody-tail mixture was analysed by SDS-PAGE.

#### **2.2.4.4 SDS-PAGE (performed by Dr. Dorota Smolarek)**

The sodium dodecyl-sulphate - polyacrylamide- gel electrophoresis (SDS-PAGE) technique was employed for the electrophoretic separation of proteins. 4-12% Bis-Tris NuPage™ pre-casted gels were used for the detection of the antibodies, and 12% Bis-Tris NuPage™ gels for the detection of IL-15. Gels were loaded into the XCell SureLock® Mini-Cell gel tank (Novex, Life Technologies) and the tank was filled with MES-SDS or MOPS running buffer, for IL-15 and for the antibodies respectively. The samples to be tested were added NuPage™ LDS loading buffer and loaded on the gel. A standard molecular size marker (Novex® Sharp unstained protein standard or Mark12™ unstained standard) was used. The gel was run at 200V, 120 mA for approximately 45 min.

#### **2.2.4.5 SDS-PAGE gel staining (performed by Dr. Dorota Smolarek)**

Silver staining for total IL-15 (tailed and untailed) protein was performed using the SilverXpress® Silver Staining kit following a protocol as per manufacturer instructions. The antibodies underwent staining with the SimplyBlue™ SafeStain as per manufacturer instructions.

#### **2.2.4.6 Western Blotting (performed by Dr. Dorota Smolarek)**

Proteins separated on the SDS-PAGE gel were transferred to nitrocellulose membranes by dry-transfer using the Life Technologies' iBlot® system, then the membrane was blocked using Casein for 1 hour. The membrane was then washed 3 times with water, the primary antibody was applied (diluted in PBS) and incubated at room temperature for 1 hour. The membrane was washed again 3 times in water and the secondary antibody was applied (diluted in PBS). The membrane was washed again and the blot was developed using SIGMAFAST™ 3,3'-diaminobenzadine tablets as per manufacturer's protocol.

#### **2.2.4.7 Fluorescein amidite (FAM) labelling of the tail (performed by Dr. Dorota Smolarek)**

The tail was FAM labelled using the AnaTag™ kit in order to confirm protein tailing. The protocol followed was as per manufacturer's instructions; briefly 230µg of the tail was mixed with FAM dye, reconstituted in DMSO, at a 1:2 tail to dye molar ratio and the mixture was incubated rotating for 3 hours. Then the unconjugated dye was removed by dialysis against PBS, changing the buffer twice, finally the mixture of tail with FAM dye was concentrated to 100µl using 3K cut off Amicon Ultra-0.5 Centrifugal Filter Unit (Merc Millipore). The tail was then ready to be used in protein modification. The SDS-PAGE gels were run as described above, and the bands were visualised under UV light using a G:BOX gel image device (Syngene, UK).

### 2.2.5 *In vivo* experiments

All experiments were performed in accordance with the UK Home Office Animals Scientific Procedures Act 1986 under Project Licence Number (PPL): 70/7743 and Personal Licence Number (PIL): 70/24581

#### 2.2.5.1 Subcutaneous implantation of PCa cells on C57BL/6 mice

Inbred male 6-8 weeks old C57BL/6 JAX strain mice (from The Jackson Laboratory, UK) were subcutaneously injected with  $5 \times 10^6$  TRAMP-C2 cells in 100µl PBS into the left flank under isoflurane anaesthesia in 1% N<sub>2</sub>O, 2% O<sub>2</sub>. Tumour dimensions were monitored daily or every other day, when the tumour was palpable it was measured using calipers and the tumour volume was calculated using the formula where only one dimension is known  $= \frac{\pi}{6} L^3$ .

#### 2.2.5.2 Subcutaneous injection of the cocktail of drugs (IL-15 and two antibodies)

On the day that the tumours on mice were palpable (between 30-40mm<sup>3</sup>), the mice were divided in three groups (10 mice in each group) and injected with the sterile-filtered cocktail of drugs. Each mouse was injected subcutaneously in the close vicinity of the tumour with either 100µl of sterile-filtered PBS (group 1), or with 100µl of a cocktail of untailed drugs: 5µg IL-15 and 100µg of each antibody, prepared in PBS (group 2) or with the tailed version of the cocktail of drugs (group 3) at the same concentrations in PBS (5µg IL-15, and 100µg of each antibody). The mice were injected only once and by day 73 they were all culled.

### 2.2.6 Histological procedures

At the time that the mice were culled their tumour masses were extracted and preserved in Optimal Cutting Temperature (OCT) cryo-embedding medium and at liquid nitrogen until further use. Tissue sections of 2µm were cut using a sledge microtome (Leica, UK) and transferred onto Polysine™ slides and allowed to air-dry overnight. They were stored at -80°C, if not used immediately.



### **2.2.6.1 Haematoxylin & Eosin (H&E) staining**

H&E staining was performed on the tumour tissue sections from the different groups of mice; Haematoxylin stains the nuclei of cells dark blue and the cytoplasm pink by eosin.

Slides with the tissue sections were immersed in haematoxylin for 7 minutes, followed by a 15-20min wash with running water. Sections were then dipped in eosin for 5 minutes and rinsed briefly in water. They were then dehydrated by immersing through an increasing concentration of alcohol (90%, 100%). Finally, sections were cleared in xylene and mounted in DPX (dibutyl phthalate containing xylene).

### **2.2.6.2 Immunofluorescence staining**

The slides with the tissue sections were removed from the -80°C and air dried for 20min. Cold acetone was poured on the slides which were stored at -20°C for 10min. They were air dried again for 20min. Wax pen was applied around the tissue section, which was then blocked with 1x casein blocking buffer for 1 hour. If the antibody of interest was biotinylated, streptavidin was applied for 15min, washed twice with PBS, biotin was applied for 15min and the slides were washed twice again with PBS. Fc block was then added and incubated for one hour. The primary antibody was applied on the slides which were incubated for 1 hour, followed by washing three times with PBS and application of the secondary antibody, and incubation for another 1 hour. Both antibodies were diluted in blocking buffer and 1% BSA. The slides were washed three times with PBS and Vectashield mounting medium was applied. The slides were viewed using an Olympus BX51 microscope (Olympus Optical, London, UK).

### 2.2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., USA). The appropriate significance testing (i.e. one-way ANOVA) with a 5% confidence level was used according to the type of data and results were presented as the mean  $\pm$  standard error of the mean (s.e.m.).

### CHAPTER 3

Design of an allogeneic prostate tumour cell – immune effector cell  
coculture model for screening of anti-tumour efficacy of  
immunotherapeutic cytokines

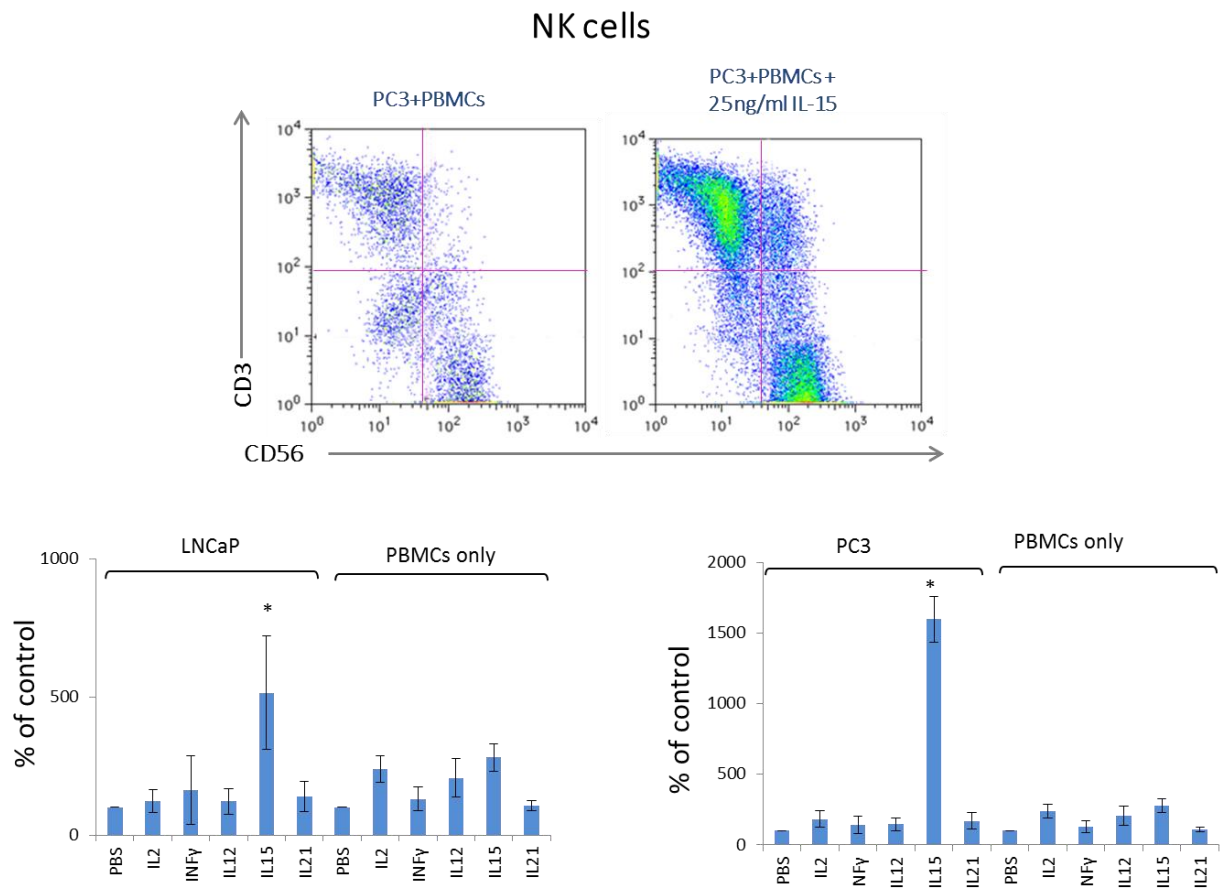
### 3.1 Introduction

The PCa microenvironment is a highly immunosuppressive and poorly immunogenic environment, where the infiltrating immune cells become anergic or suppressive. The aim of this project was to overcome this immunosuppression by enhancing the immune effector cells. In order to mimic this microenvironment it was critical to design *in vitro* and *in vivo* models of the prostate tumour –immune effector cell interaction and tumour cell killing that would mimic the prostate tumour microenvironment and would enable us to identify, test and optimize novel immunotherapeutics. Two models were designed in this project, an allogeneic and a syngeneic (in order to investigate any alloreactivity effects), where immune effector cells were cocultured with PCa cell lines, in the presence of a panel of Th1 cytokines; the expansion and activity (target cell killing) of the immune effector cells in both the allogeneic and syngeneic coculture models were investigated.

Regarding the allogeneic coculture model, the non-adherent fraction of PBMCs from healthy blood donors, was cocultured with PC-3 and LNCaP cell lines, whereas in the syngeneic coculture model, NK- or T- purified cells isolated from splenocytes of C57BL/6 mice, were cocultured with the TRAMP-C1 cell line, which is also an isolated cell line from the prostate of a C57BL/6 mouse with transgenic adenocarcinoma. In both coculture models, a panel of therapeutic cytokines were studied and included IL-2, IL-12, IL-15, IL-21 and IFN $\gamma$ , due to their clinical and therapeutic implication.

Preliminary data to this project have shown that in an allogeneic coculture system of immune effector cells with PCa cell lines (PC-3 and LNCaP) IL-15 is the most potent activator of NK cell and of CD8 T cell expansion, as observed by CD56<sup>+</sup> CD3<sup>-</sup> cell expression (Figure 3.1) and CD8<sup>+</sup> CD4<sup>-</sup> cell expression (Figure 3.2) respectively (n=5, \*p<0.01, one-way ANOVA). The effects of IL-15 were compared to control (no cytokine) or other therapeutic Th1 cytokines including IL-2, IFN $\gamma$ , IL-12 and IL-21. IL-15 also increased the perforin expression by NK and CD8 T cells as well as the killing of PCa cells, as detected by Annexin<sup>+</sup> PI<sup>+</sup> PCa cell expression (n=5, \*p<0.001, one-way ANOVA) (Figure 3.3). IL-15 did not affect the T-regulatory cell expansion, as observed by CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> T cell expression, when compared to control or the other cytokines, whereas IL-2 highly expanded them (n=5, \*p<0.05, \*\*p<0.01, 1-way

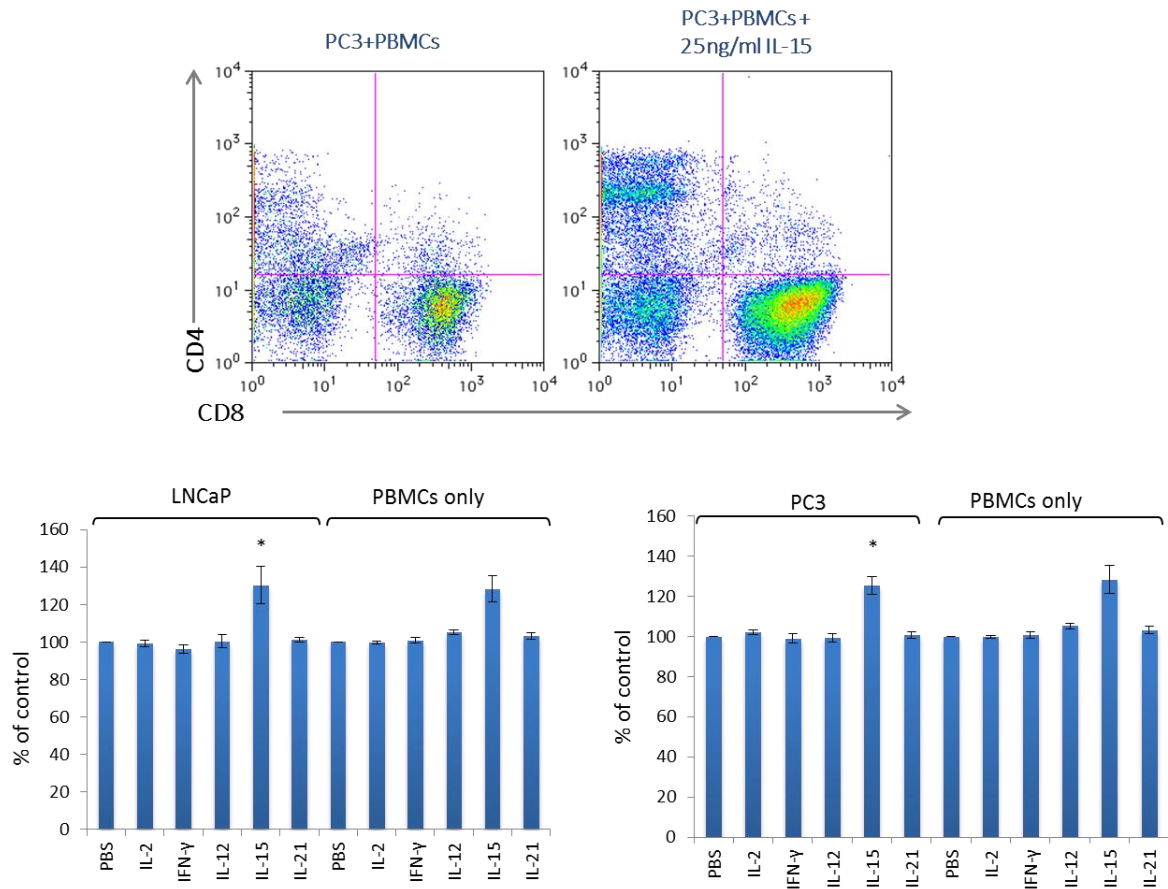
ANOVA) (Figure 3.4). The ratio of immune effector cells to target cells (E:T) at the allogeneic coculture model that has been used to generate these preliminary data, was 8:1 ( $1.6 \times 10^6$ /ml to  $2 \times 10^5$ /ml)(Figures 3.1-3.4) (Elhage, Galustian et al. 2011).



**Figure 3.1: CD3 and CD56 cell staining in cocultures of non-adherent PBMCs and PC-3 or LNCaP cells.**

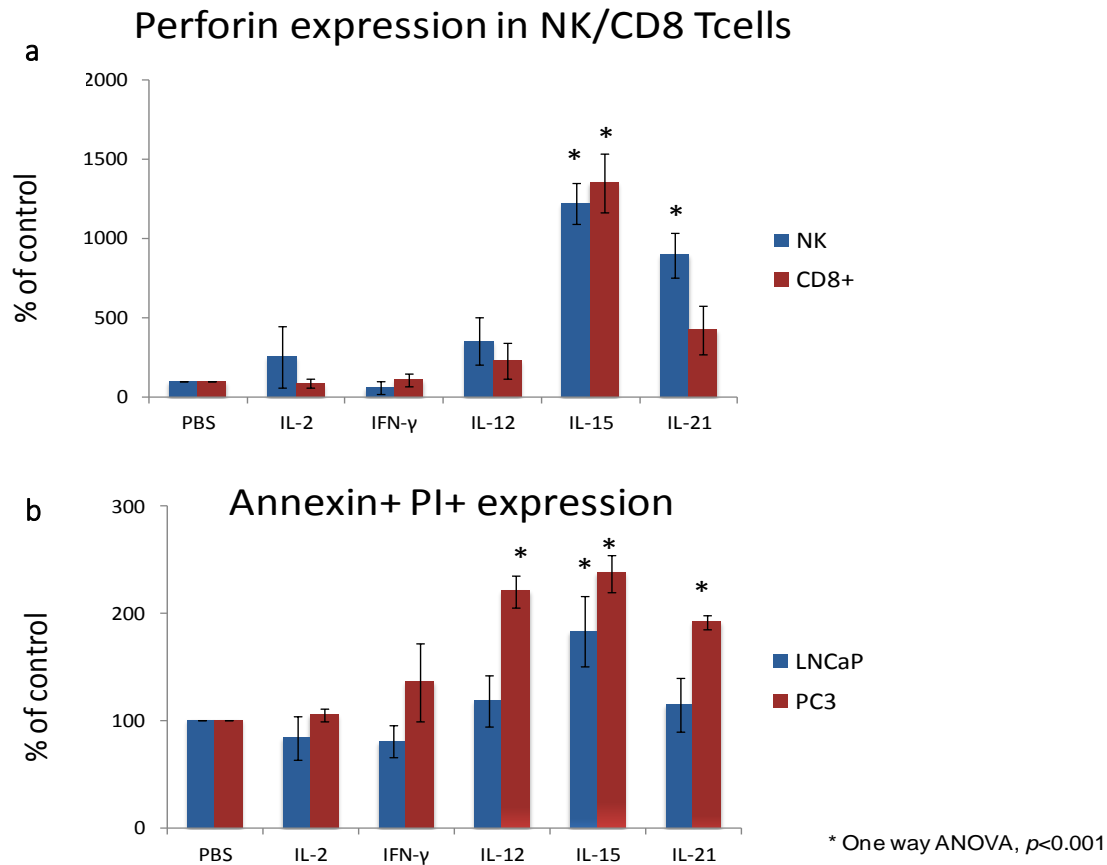
The NK cell expansion, as observed by CD56<sup>+</sup> CD3<sup>-</sup> cell staining, within the non-adherent fraction of PBMCs is shown, in the absence or presence of LNCaP or PC-3 cells, in the presence or absence of a panel of therapeutic Th1 cytokines (10ng/ml IL-2, 25ng/ml IFN $\gamma$ , 25ng/ml IL-12, 25ng/ml IL-15, 25ng/ml IL-21). The dotplots are showing the NK cell expansion in the absence or presence of 25ng/ml IL-15 (n=5, \*p<0.01, one-way ANOVA).

## CD8 cells



**Figure 3.2: CD4 and CD8 cell staining in cocultures of non-adherent PBMCs and PC-3 or LNCaP cells.**

The CD8 T cell expansion, as observed by CD8 and CD4 markers' staining, within the non-adherent fraction of PBMCs is shown, in the absence or presence of LNCaP or PC-3 cells, in the presence or absence of a panel of therapeutic Th1 cytokines (10ng/ml IL-2, 25ng/ml IFN $\gamma$ , 25ng/ml IL-12, 25ng/ml IL-15, 25ng/ml IL-21). The dotplots are showing the CD8<sup>+</sup> cell expansion in the absence or presence of 25ng/ml IL-15 (n=5, \*p<0.01, one-way ANOVA).



**Figure 3.3: Perforin and Annexin/PI staining of the NK and CD8 T cells and of the PC-3 and LNCaP cells respectively.** a) **Perforin staining of the NK and CD8 T cells:** The perforin expression by NK and CD8 T cells in the coculture models of non-adherent PBMCs and PC-3 cells, in the presence of a panel of therapeutic Th1 cytokines is shown. b) **Annexin/PI staining of the PC-3 and LNCaP cells.** The percentage expression of Annexin<sup>+</sup> and PI<sup>+</sup> PC-3 or LNCaP cells is shown, when the cells were cocultured with non-adherent PBMCs in the presence of the Th1 therapeutic cytokines (10ng/ml IL-2, 25ng/ml IFN $\gamma$ , 25ng/ml IL-12, 25ng/ml IL-15, 25ng/ml IL-21) (n=5, \* $p < 0.001$ , one-way ANOVA).

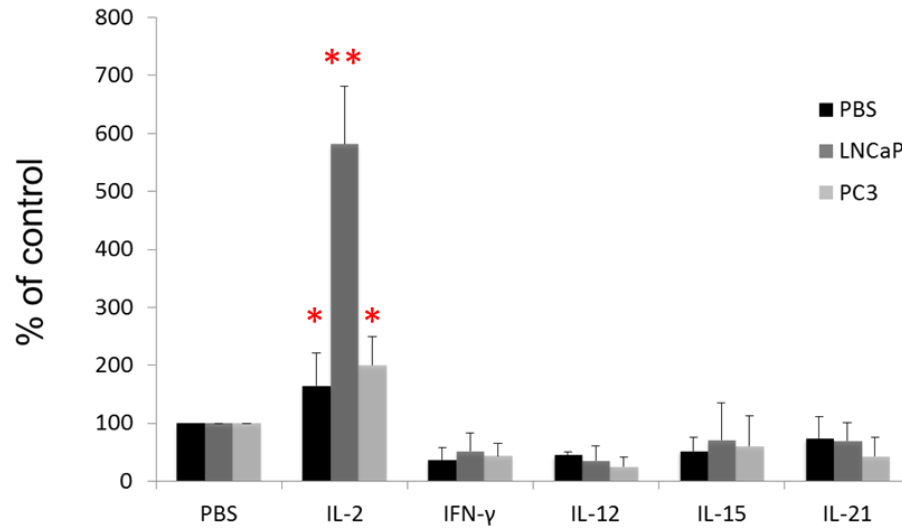
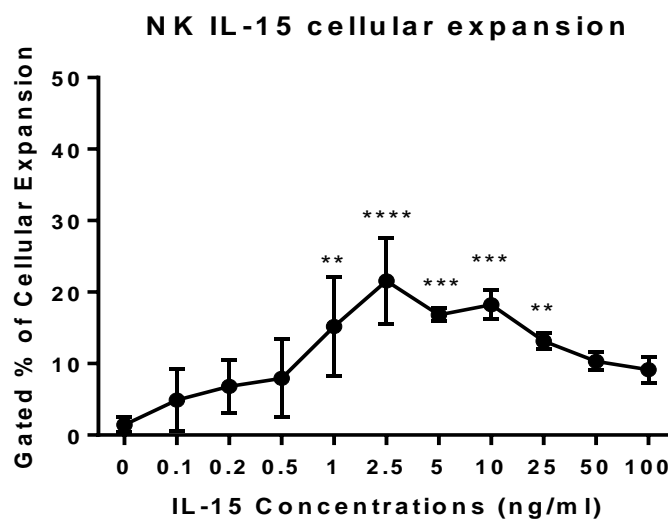


Figure 3.4: T regulatory cell ( $CD4^+$   $CD25^+$   $FoxP3^+$ ) expansion in cocultures of non-adherent PBMCs and PCa cells. Non-adherent PBMCs were cultured either alone (PBS) or in the presence of PC-3 or LNCaP cells, in the presence or absence of a panel of therapeutic Th1 cytokines (10ng/ml IL-2, 25ng/ml IFN $\gamma$ , 25ng/ml IL-12, 25ng/ml IL-15, 25ng/ml IL-21) (n=5, \*p<0.05, \*\*p<0.01, 1-way ANOVA).



In a separate study from our group, Ms Fiona Watkinson, cultured non-adherent PBMCs in the presence of varying IL-15 concentrations (0.1-100 ng/ml), and investigated at which IL-15 concentration NK cells were expanding the most. She demonstrated that IL-15 induces the highest NK cell expansion at a concentration of 2.5ng/ml and at higher concentrations NK cells show a decreased expansion (n=5, \*= P<0.05, \*\*= P<0.005, \*\*\*=P<0.0005 and \*\*\*\*=P<0.0001, 1-way ANOVA) (Figure 3.5).



**Figure 3.5:** NK cell (CD56<sup>+</sup> CD3<sup>-</sup>) expansion in response to different concentrations of IL-15. The expansion of NK cells at varying concentrations of IL-15 is shown, when non-adherent PBMCs were cultured alone (n=5, \*= P<0.05, \*\*= P<0.005, \*\*\*=P<0.0005 and \*\*\*\*=P<0.0001, 1-way ANOVA).

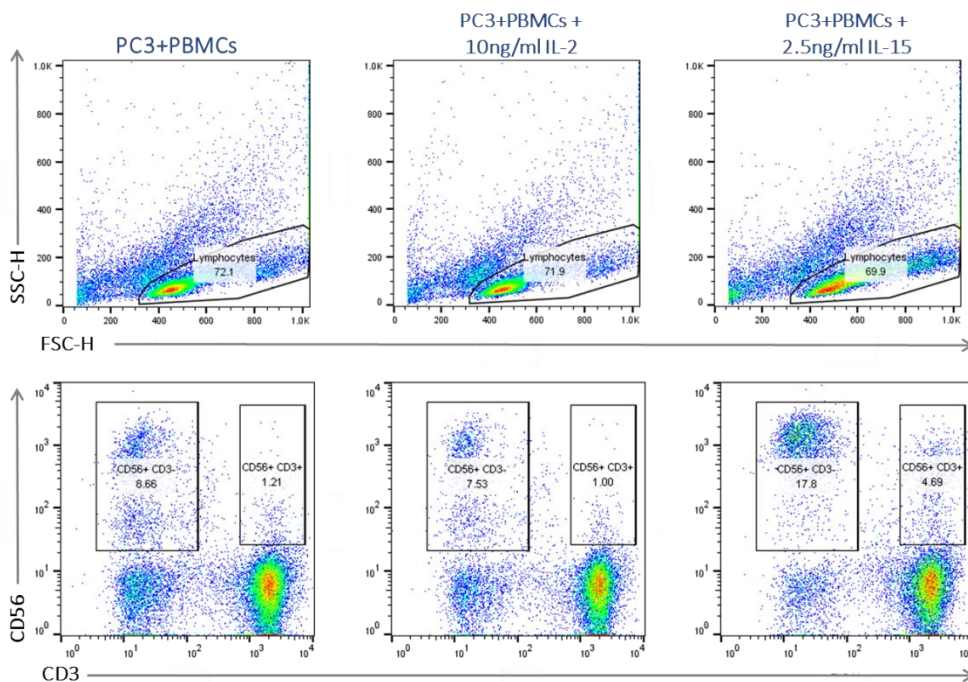
In pre-existing literature however, different E:T ratios have been used (Marten, Renoth et al. 2001, Balsamo, Vermi et al. 2012, Shi, Fan et al. 2014), therefore a critical step before progressing further with experiments, was to investigate which E:T ratio would produce the greatest expansion of NK, NKT and CD8 T cells, and which would enhance their cytotoxicity towards the PCa cells.

## 3.2 Results

### 3.2.1 Optimisation of the immune effector cell- target cell ratio in the allogeneic coculture model to obtain maximum NK, NKT and CD8 T cell expansion.

In order to optimise the allogeneic coculture's E:T ratio, non-adherent PBMCs were isolated and cultured either alone or cocultured with the PC-3 cell line at 8:1, 4:1 and 2:1 ratios in the presence or absence of IL-2, IFN $\gamma$ , IL-12, IL-15 and IL-21 at their ED50 doses. 7 days after incubation, the non-adherent PBMCs were fluorescently labelled for the markers CD56, CD3, CD8, perforin (protein expressed in the cytotoxic granules released by NK and CD8<sup>+</sup> T cells) and CD107a (marker for degranulation of NK and CD8<sup>+</sup> T cells).

Figure 3.6 shows the FACS dotplots and the gating strategy when non-adherent PBMCs were cocultured with PC-3 cells, either in the absence of cytokines (control) or in the presence of 10ng/ml IL-2 or 2.5ng/ml IL-15. As shown, in the presence of IL-15, NK and NKT cells expand more, when compared to control or IL-2.



**Figure 3.6: Gating strategy followed when non-adherent PBMCs were cocultured with PC-3 cells.** The top panel dotplots show the gating of lymphocytes using forward and side scatter. The bottom panel shows the NK (CD56<sup>+</sup> CD3<sup>-</sup>) and NKT (CD56<sup>+</sup> CD3<sup>+</sup>) cell gating from the lymphocyte population, when non-adherent PBMCs were cocultured with PC-3 cells either alone or in the presence of 10ng/ml IL-2 or 2.5ng/ml IL-15.

Figure 3.7a-c shows the NK cell expansion, as measured by CD56<sup>+</sup> CD3<sup>-</sup> cell expression at the different E:T coculture ratios; Figure 3.8d-f shows the perforin expression by NK cells and Figure 3.9g-i shows the CD107a<sup>+</sup> expression by NK cells.

NK cells were highly expanded in the presence of IL-15, compared to control and the other Th1 cytokines, when non-adherent PBMCs were cultured alone, however in cocultures with PC-3 cells at 8:1 and 4:1 E:T ratios the expansion of NK cells by IL-15 was even greater. Perforin and CD107a expressions by NK cells were also higher in the presence of IL-15 compared to control or the other cytokines, at 8:1 and 4:1 E:T ratios in regards to perforin and at 8:1 in regards to CD107a (n=5, \*p<0.05, \*\*p<0.01, 1-way ANOVA). The cytokines IL-2, IFN $\gamma$ , IL-12 and IL-21 have little or no effect on the expansion of NK cells in this model.

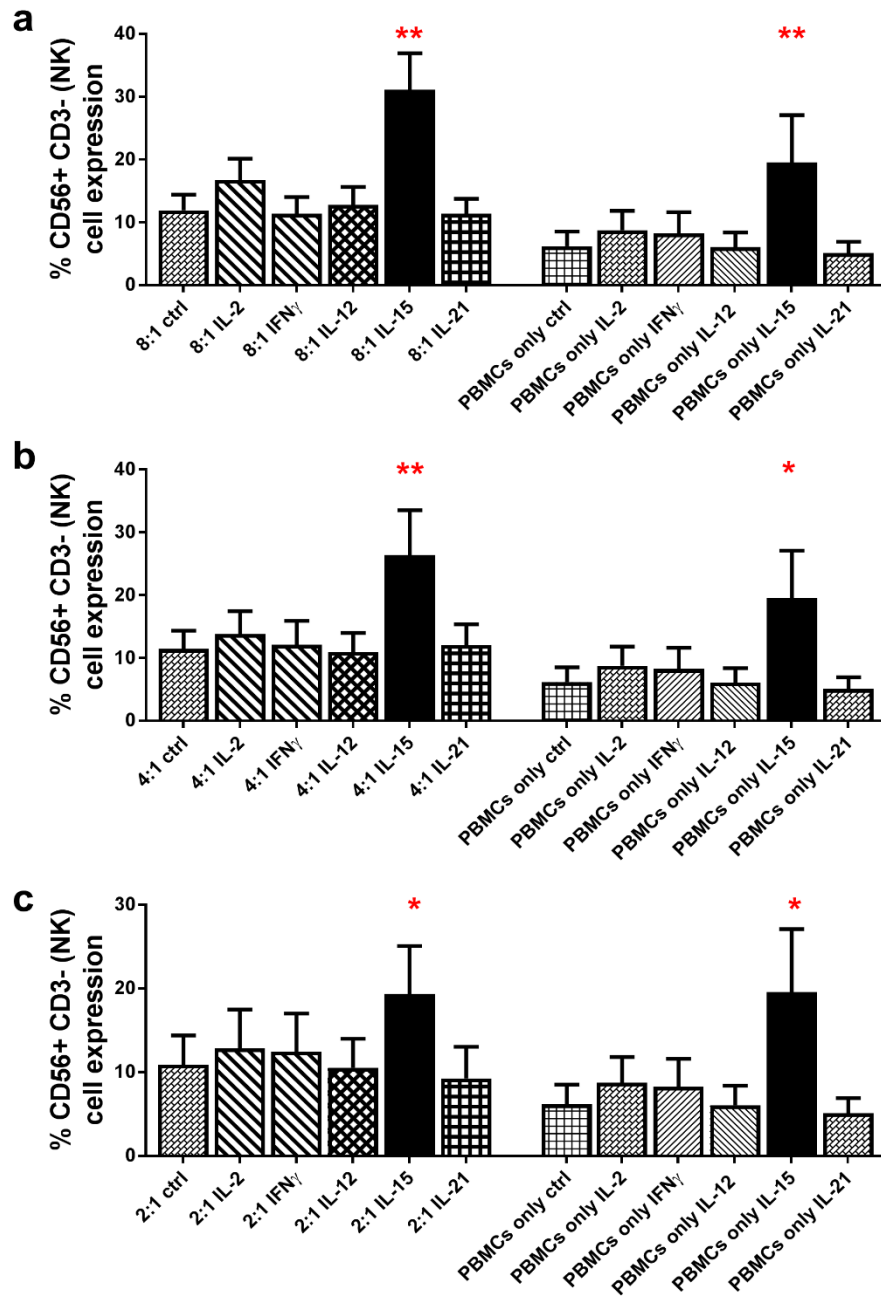


Figure 3.7: NK cell expansion at different coculture ratios of non-adherent PBMCs and PC-3 cells. The expansion of NK cells is shown, as measured by CD56<sup>+</sup> CD3<sup>-</sup> cell expression, in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21), when non-adherent PBMCs were cultured alone or in the presence of PC-3 cell line at **a) 8:1**, **b) 4:1** and **c) 2:1** E:T ratios (n=5, \*p<0.05, \*\*p<0.01, 1-way ANOVA).

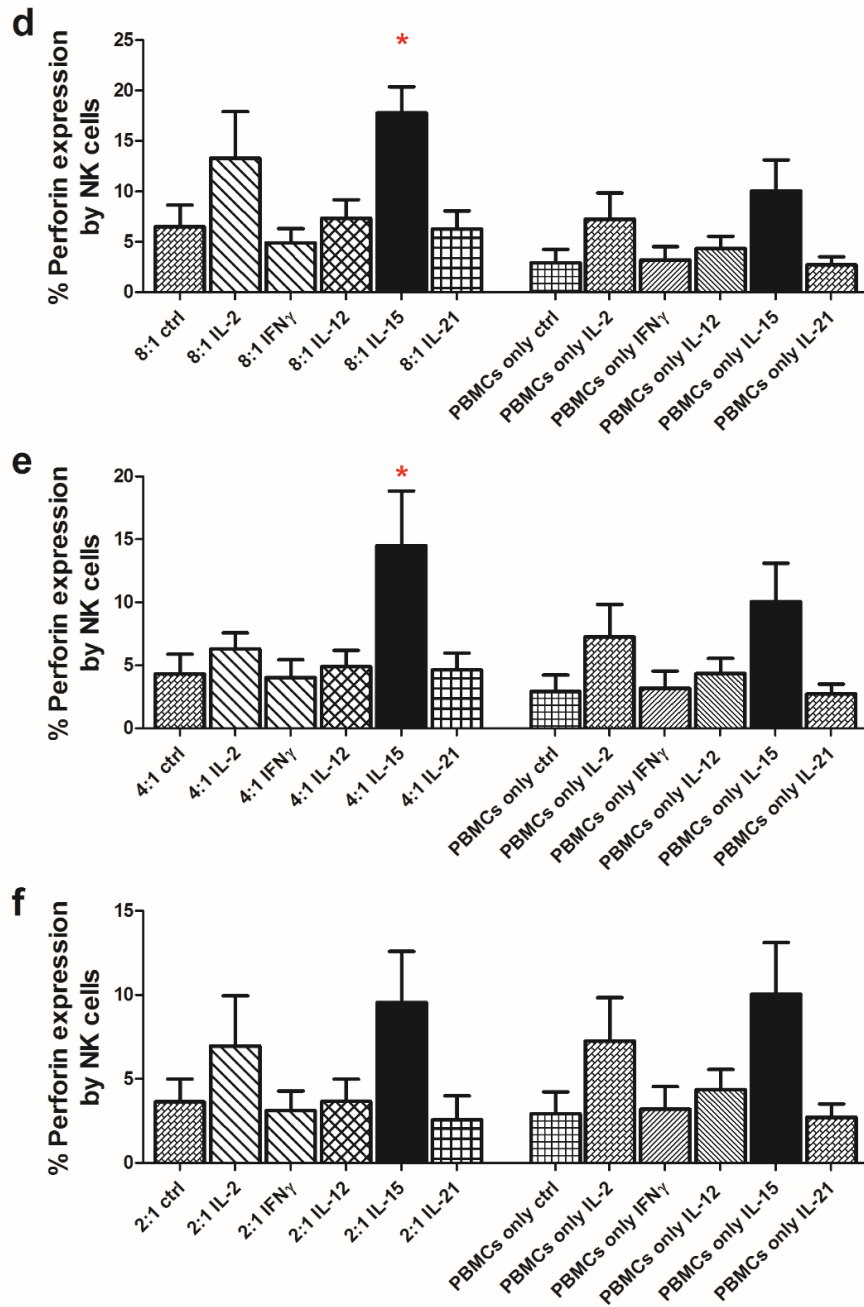


Figure 3.8: Perforin expression by NK cells at different coculture ratios of non-adherent PBMCs and PC-3 cells. The perforin expression by NK cells in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21) is shown, when non-adherent PBMCs were cultured alone or in the presence of PC-3 cell line at **d)** 8:1, **e)** 4:1 and **f)** 2:1 E:T ratios (n=5, \*p<0.05, 1-way ANOVA).

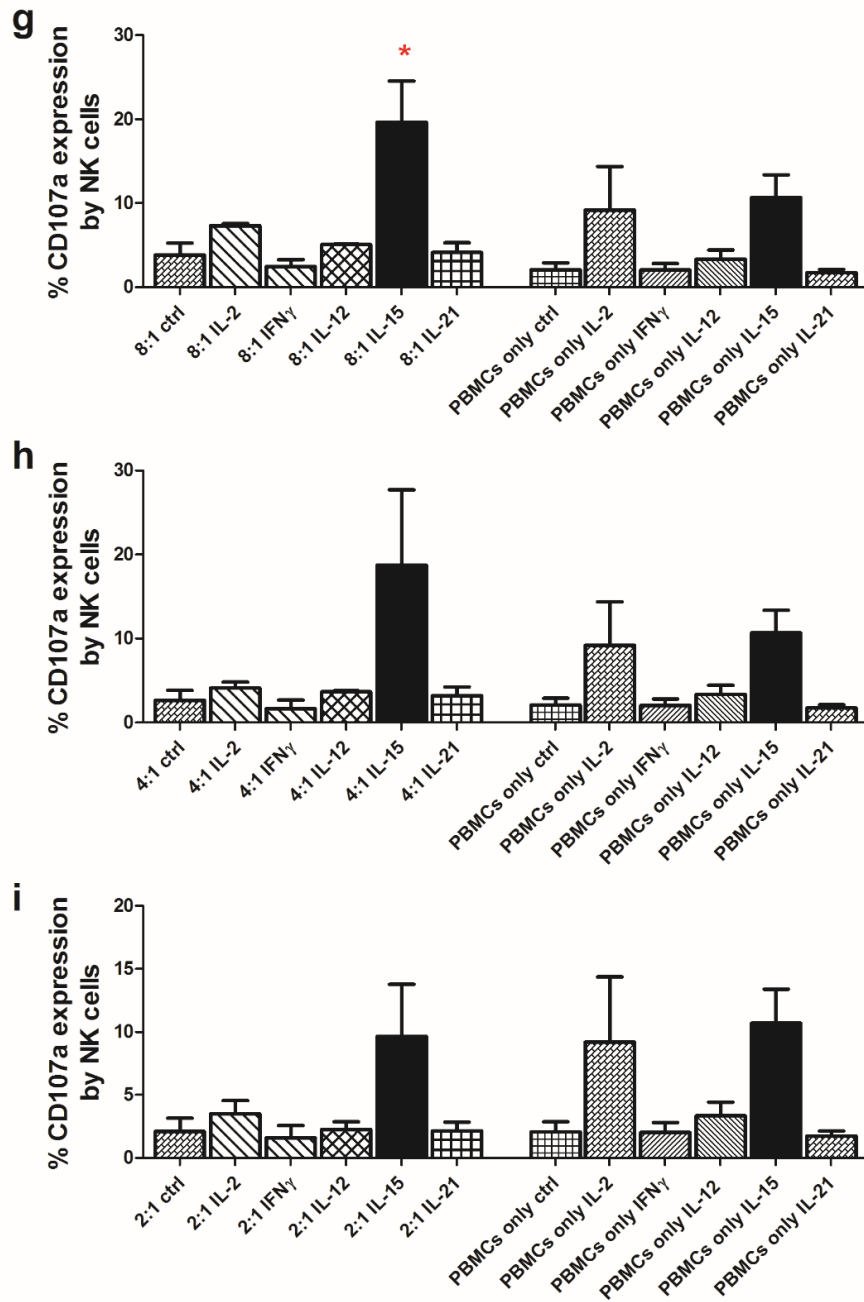
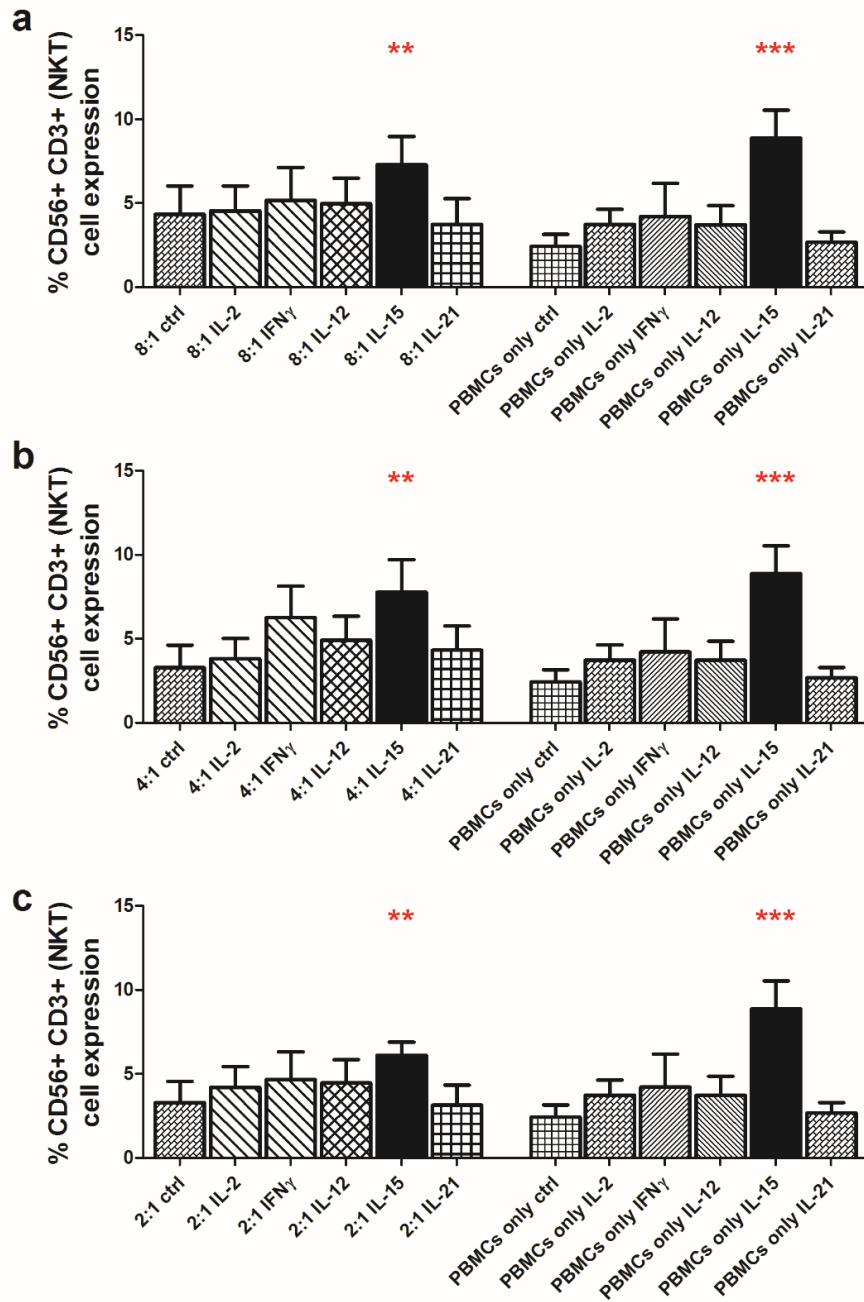


Figure 3.9: CD107a expression by NK cells at different coculture ratios of non-adherent PBMCs and PC-3 cells. The CD107a<sup>+</sup> expression by NK cells is shown, in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21), when non-adherent PBMCs were cultured alone or in the presence of PC-3 cell line at **g**) 8:1, **h**) 4:1 and **i**) 2:1 E:T ratios (n=5, \*p<0.05, 1-way ANOVA).

Figures 3.10-3.12 show the NKT cell expansion, perforin and CD107a expression by NKT cells.

The presence of IL-15 highly expanded the NKT cells, as observed by CD56<sup>+</sup> CD3<sup>+</sup> cell expression, when the non-adherent PBMCs were cultured alone or in the presence of PC-3 cell line at all E:T ratios (n=5, \*\*p<0.01, \*\*\*p<0.001, 1-way ANOVA). No other cytokine expanded the NKT cells. Perforin and CD107a expression by NKT cells were also increased in the presence of IL-15, compared to control or the other cytokines, however only when the PBMCs were cultured alone (n=5, \*p <0.05, \*\*p<0.01, \*\*\*p<0.001, 1-way ANOVA). In the presence of the PC-3 cell line, the perforin and CD107 expressions by NKT cells were not increased by the presence of the cytokines, when compared to control (n=5, p>0.05, 1-way ANOVA).



**Figure 3.10: NKT cell expansion at different coculture ratios of non-adherent PBMCs and PC-3 cells.** The expansion of NKT cells is shown, as measured by CD56<sup>+</sup> CD3<sup>+</sup> cell expression, in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21), when non-adherent PBMCs were cultured alone or in the presence of PC-3 cell line at **a) 8:1**, **b) 4:1** and **c) 2:1** E:T ratios (n=5, \*\*p<0.01, \*\*\*p<0.001, 1-way ANOVA).



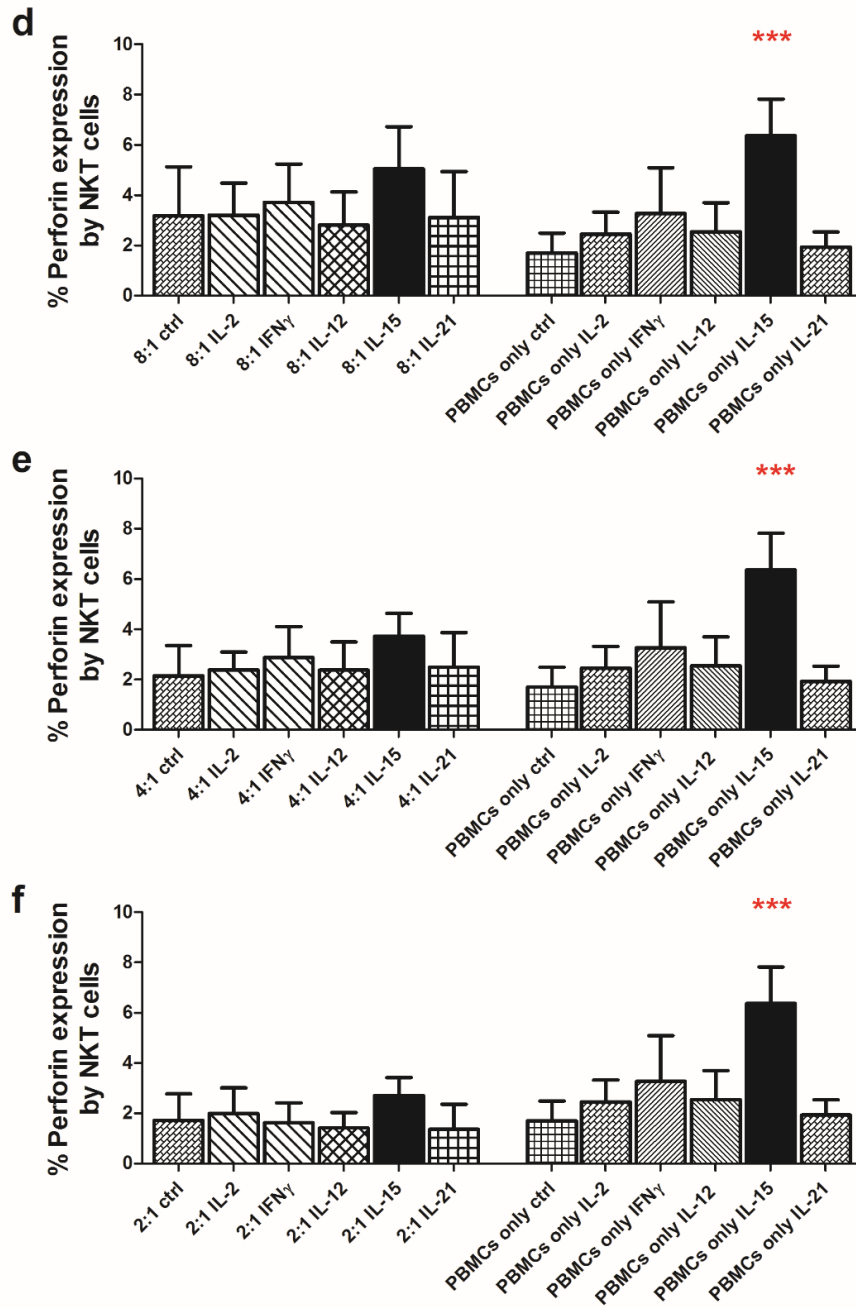
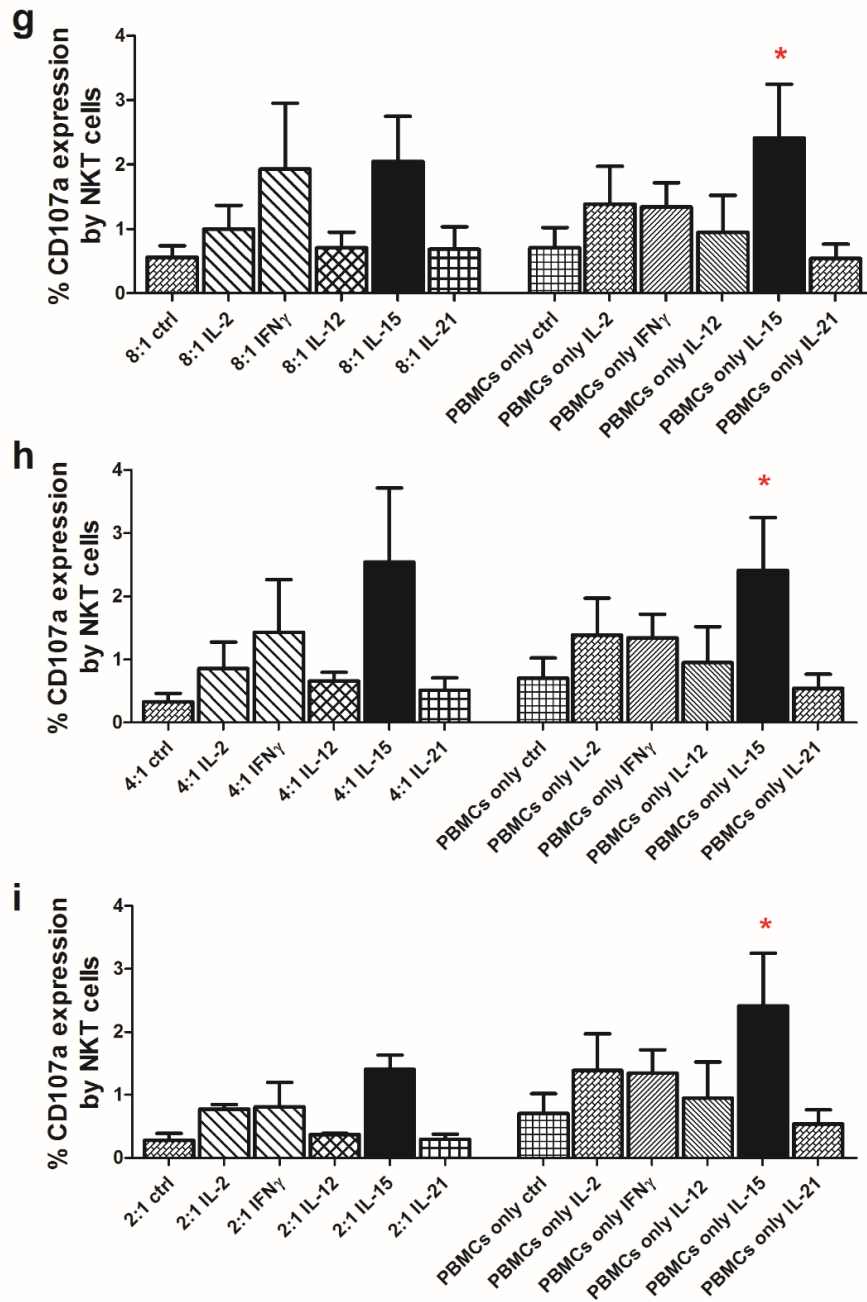
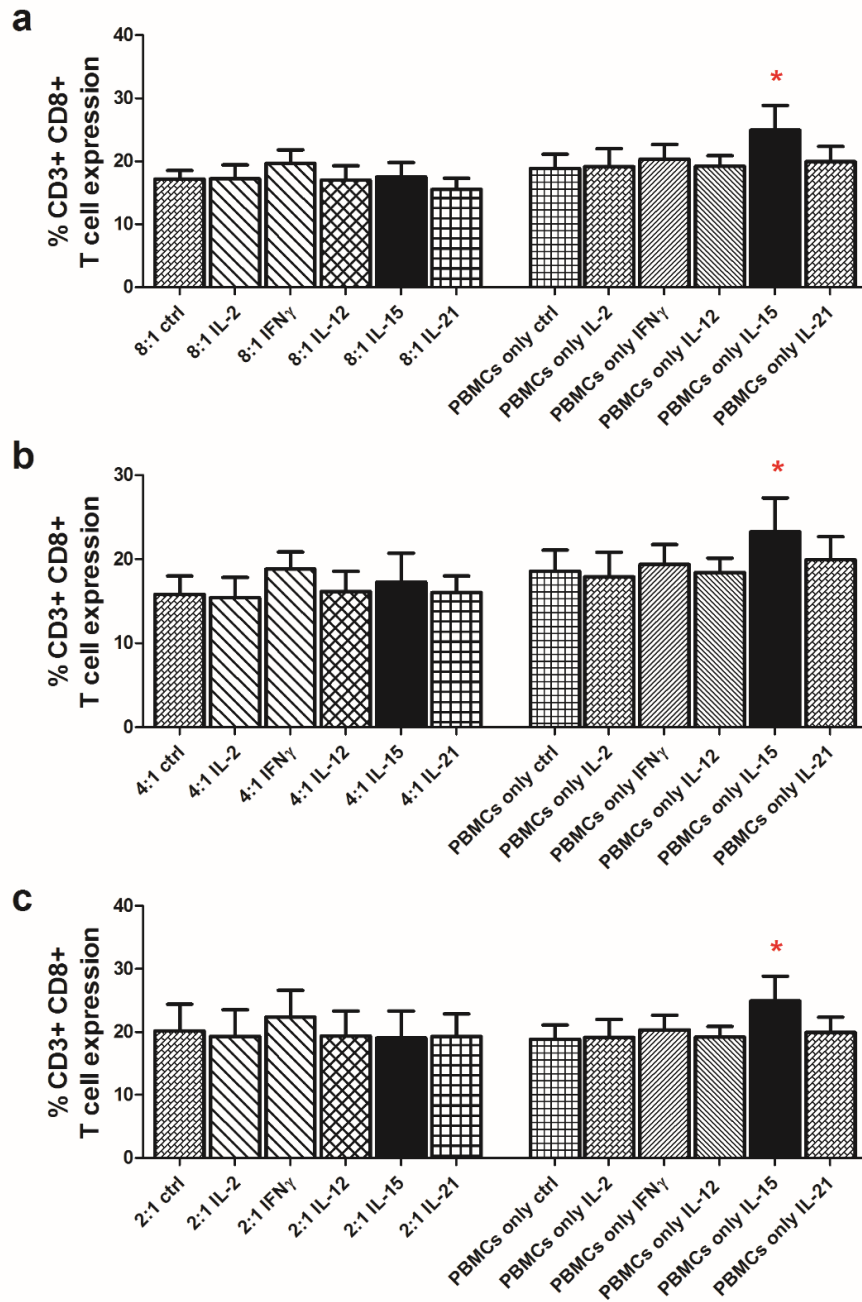


Figure 3.11: Perforin expression by NKT cells at different coculture ratios of non-adherent PBMCs and PC-3 cells. The perforin expression by NKT cells is shown, in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21), when non-adherent PBMCs were cultured alone or in the presence of PC-3 cell line at **d)** 8:1, **e)** 4:1 and **f)** 2:1 E:T ratios (n=5, \*\*\*p<0.001, 1-way ANOVA).



**Figure 3.12: CD107a expression by NKT cells at different coculture ratios of non-adherent PBMCs and PC-3 cells** The CD107a<sup>+</sup> expression by NKT cells is shown, in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21), when non-adherent PBMCs were cultured alone or in the presence of PC-3 cell line at **g)** 8:1, **h)** 4:1 and **i)** 2:1 E:T ratios (n=5, \*p<0.05, 1-way ANOVA).

Figures 3.13-3.15a-i show the CD8<sup>+</sup> T cell expansion and their corresponding perforin and CD107a expression. CD8 T cells are expanded in the presence of IL-15, when non-adherent PBMCs are cultured alone, however no significant difference was observed in the cocultures of PBMCs with PC-3 cells (n=5, p>0.05, 1-way ANOVA).



**Figure 3.13: CD8 T cell expansion at different coculture ratios of non-adherent PBMCs and PC-3 cells.** The expansion of CD3<sup>+</sup> CD8<sup>+</sup> T cells is shown, in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21), when non-adherent PBMCs were cultured alone or in the presence of PC-3 cell line at **a)** 8:1, **b)** 4:1 and **c)** 2:1 E:T ratios (n=5, \*p<0.05, 1-way ANOVA).

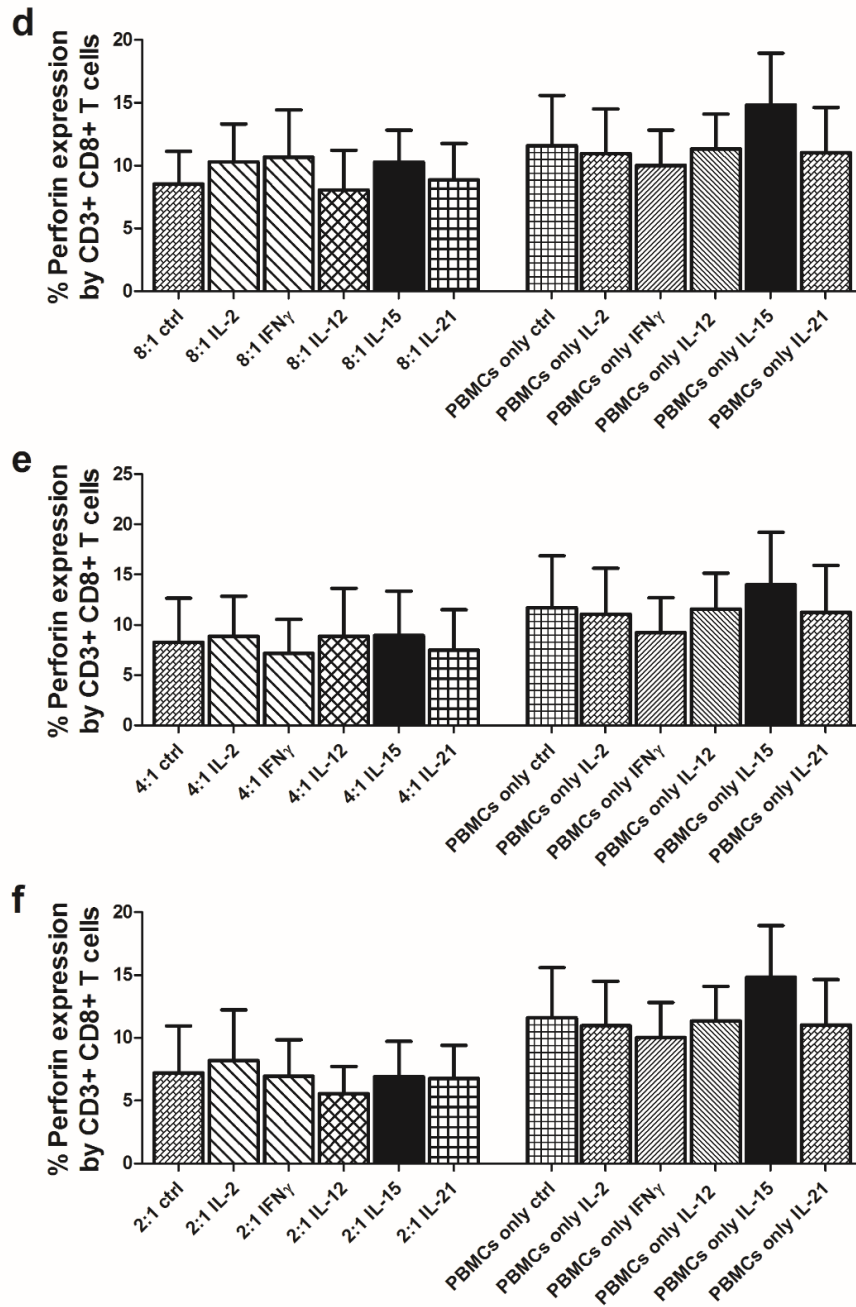


Figure 3.14: Perforin expression by CD8 T cells at different coculture ratios of non-adherent PBMCs and PC-3 cells. The perforin expression by CD3<sup>+</sup> CD8<sup>+</sup> T cells is shown, in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21), when non-adherent PBMCs were cultured alone or in the presence of PC-3 cell line at **d**) 8:1, **e**) 4:1 and **f**) 2:1 E:T ratios (n=5, p>0.05, 1-way ANOVA).

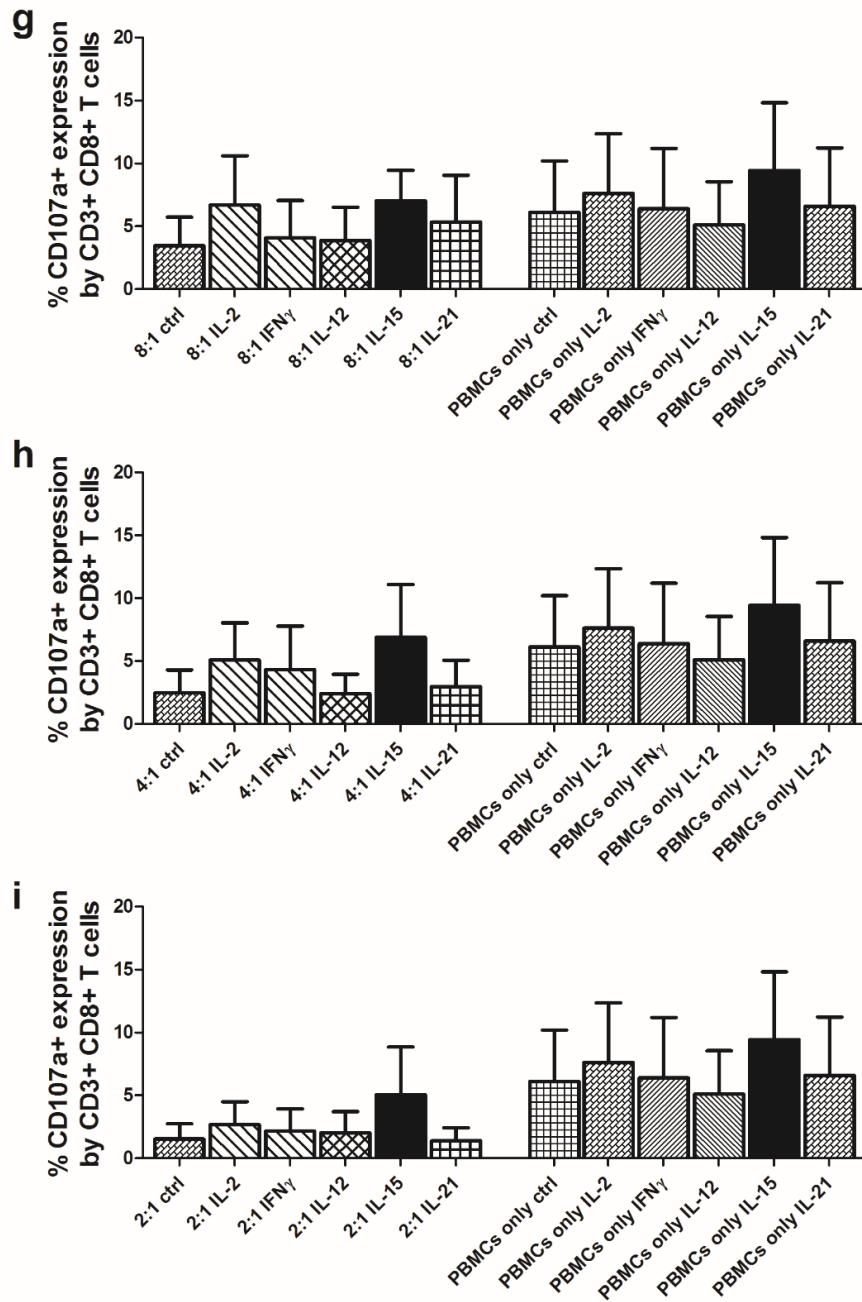


Figure 3.15: CD107a expression by CD8 T cells at different coculture ratios of non-adherent PBMCs and PC-3 cells. The CD107a<sup>+</sup> expression by CD3<sup>+</sup> CD8<sup>+</sup> T cells is shown, in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21), when non-adherent PBMCs were cultured alone or in the presence of PC-3 cell line at **g)** 8:1, **h)** 4:1 and **i)** 2:1 E:T ratios (n=5, p>0.05, 1-way ANOVA).

The same set of experiments was repeated with the LNCaP cell line. Similar results were obtained as in the cocultures with PC-3 cells, hence only six representative figures (Figure 3.16a-f) are shown, where NK and NKT cell expansion is presented.

NK and NKT cells were highly expanded in the presence of IL-15, when non-adherent PBMCs were cultured alone, when compared to control and the other Th1 cytokines. Additionally, the presence of LNCaP cells further increased the NK cell expansion at all coculture ratios in the presence of IL-15, when compared to control and the other cytokines ( $n=5$ ,  $**p<0.01$ ,  $***p<0.001$ ,  $****p<0.0001$ , 1-way ANOVA); no other cytokine produced an effect on the NK and NKT cell expansion. Perforin and CD107a expression by NK cells was also highly increased by the presence of IL-15, when non-adherent PBMCs were cultured alone, as well as in cocultures with LNCaP cells (data not shown). CD8 T cells were significantly expanded by IL-15 when non-adherent PBMCs were cultured alone, when compared to control or the other cytokines, however in cocultures with LNCaP cells a trend of an increase on CD8 T cell expansion was observed which was not significant.

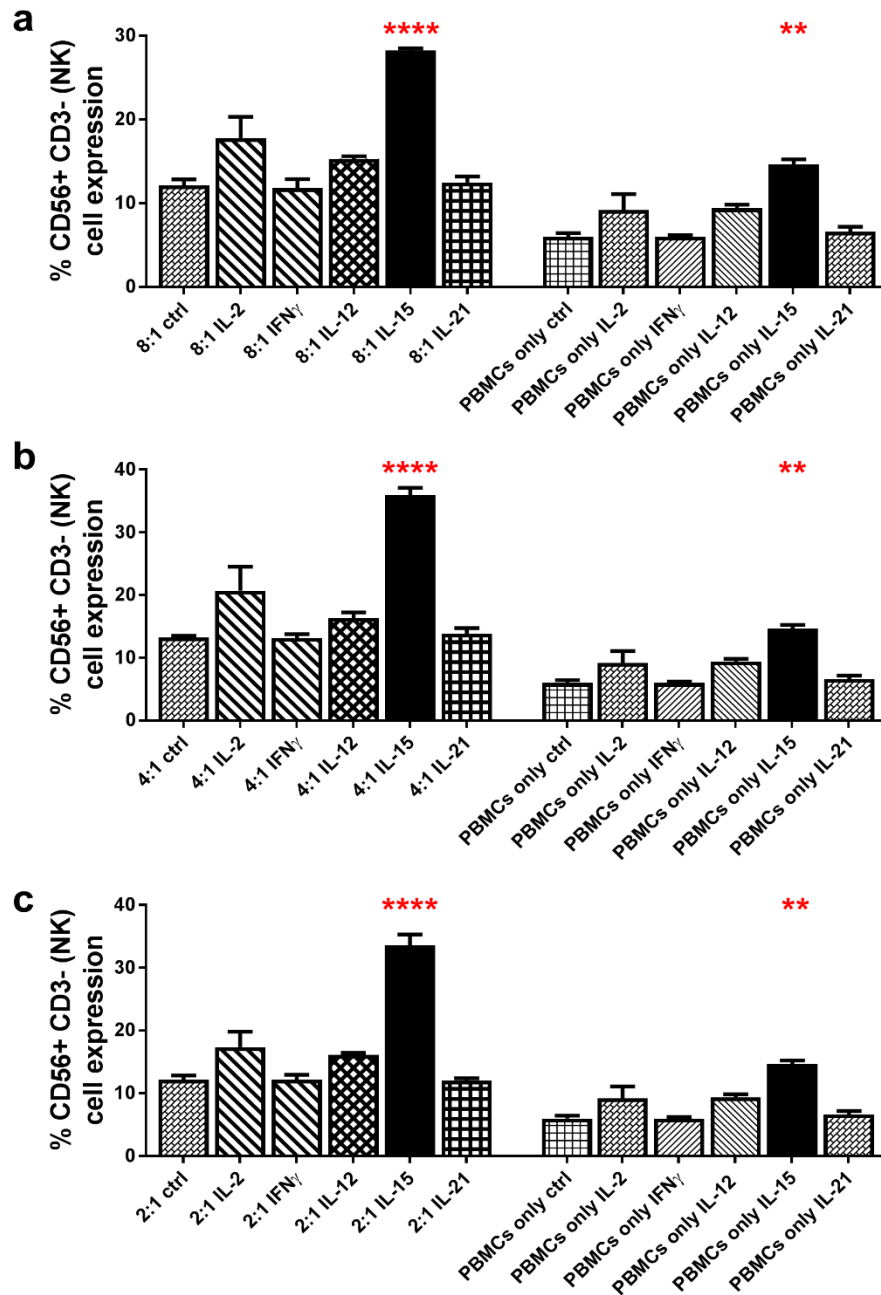


Figure 3.16: NK cell expansion at different coculture ratios of non-adherent PBMCs and LNCaP cells. The expansion of NK cells, as measured by CD56<sup>+</sup> CD3<sup>-</sup> cell expression is shown, in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21), when non-adherent PBMCs were cultured alone or in the presence of LNCaP cell line at **a) 8:1**, **b) 4:1** and **c) 2:1** E:T ratios (n=5, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, 1-way ANOVA).



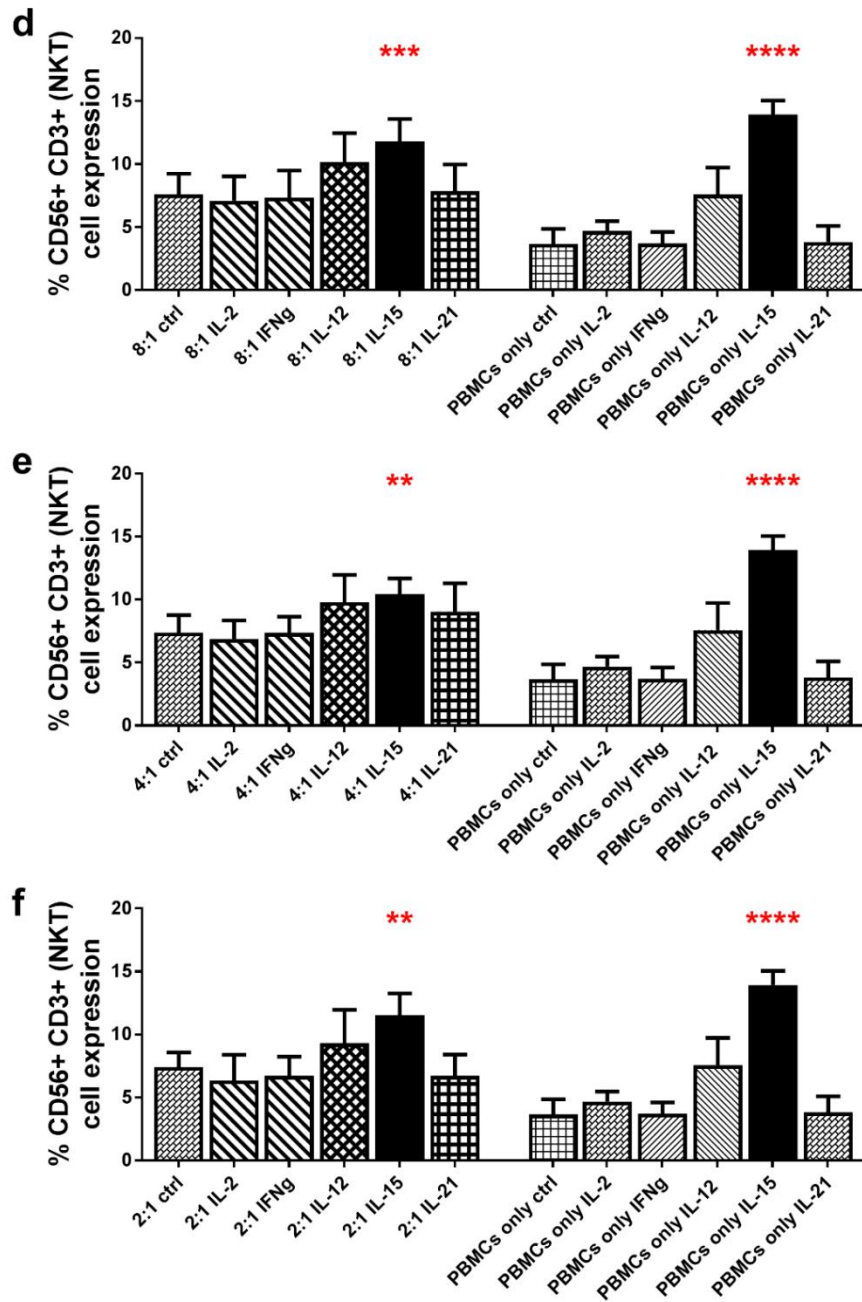


Figure 3.17: NKT cell expansion at different coculture ratios of non-adherent PBMCs and LNCaP cells. The expansion of NKT cells is shown, as measured by CD56<sup>+</sup> CD3<sup>+</sup> cell expression, in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21), when non-adherent PBMCs were cultured alone or in the presence of LNCaP cell line at **d)** 8:1, **e)** 4:1 and **f)** 2:1 E:T ratios (n=5, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, 1-way ANOVA).

### 3.2.2 Optimisation of the immune effector cell- target cell ratio on the allogeneic coculture model to obtain maximum PCa cell cytotoxicity by NK, NKT and CD8 T cells.

In order to assess the induction of the PCa cell apoptosis and necrosis by the non-adherent PBMCs and in order to observe whether the presence of IL-2 and IL-15 cytokines has any effect on the PCa cell death and at which E:T ratios and cytokine concentrations this occurred, we cultured PCa cells (PC-3 or LNCaP) at a concentration of  $1 \times 10^5$  cells/ml in a 24 well plate overnight. The following day, non-adherent PBMCs were added at E:T ratios of 20:1, 10:1, 8:1, 4:1, 2:1, 1:1 or 0.5:1 in the absence or presence of IL-2 (1 or 10ng/ml) or of various concentrations of IL-15 (0.5, 1, 2.5, 10 or 25ng/ml).

The addition of 600 $\mu$ M H<sub>2</sub>O<sub>2</sub> to one well of the plate containing PCa cells acted as a positive control. H<sub>2</sub>O<sub>2</sub> has been previously found to induce cell apoptosis and necrosis and according to the study by Kim *et al.* 2003, who investigated the mechanism of H<sub>2</sub>O<sub>2</sub> in inducing cell death in a human retinal pigment epithelial cell line (ARPE-19), H<sub>2</sub>O<sub>2</sub> at a concentration of 600 $\mu$ M causes the maximum induced cell death (Kim, Chung et al. 2003).

The percentage cell death of the PCa cells was assessed by Annexin and PI positive cell expression and it was calculated using the following equation,

$$\% \text{ specific cell death} = \left[ \frac{\% \text{ dead targets} - \% \text{ spontaneous cell death}}{\% \text{ positive control} - \% \text{ spontaneous cell death}} \right]$$

where dead targets are defined as the PCa cells derived from the cocultures of immune effector cells with PCa cells, in the presence or absence of cytokines and spontaneous cell death is defined as the PCa cells that were cultured alone, in the absence of cytokines.

When PC-3 cells were cocultured with non-adherent PBMCs at E:T ratio 2:1 the highest percentage of necrotic (PI<sup>+</sup>) and apoptotic (Annexin<sup>+</sup>) cells was observed in the presence of 10 and 25ng/ml IL-15, when compared to the percentage cell death observed in the presence of IL-2 and at 4:1 ratio in the presence of 2.5, 10 and 25ng/ml IL-15 (figure 3.18a) (n=4, \*p<0.05, 1-way ANOVA). When LNCaP cells were cocultured with non-adherent PBMCs at

E:T ratio 4:1, the highest percentage of necrotic and apoptotic cells was observed in the presence of 2.5ng/ml IL-15, when compared to the percentage cell death observed in the presence of IL-2 (figure 3.18b) (n=4, \*p<0.05, 1-way ANOVA).

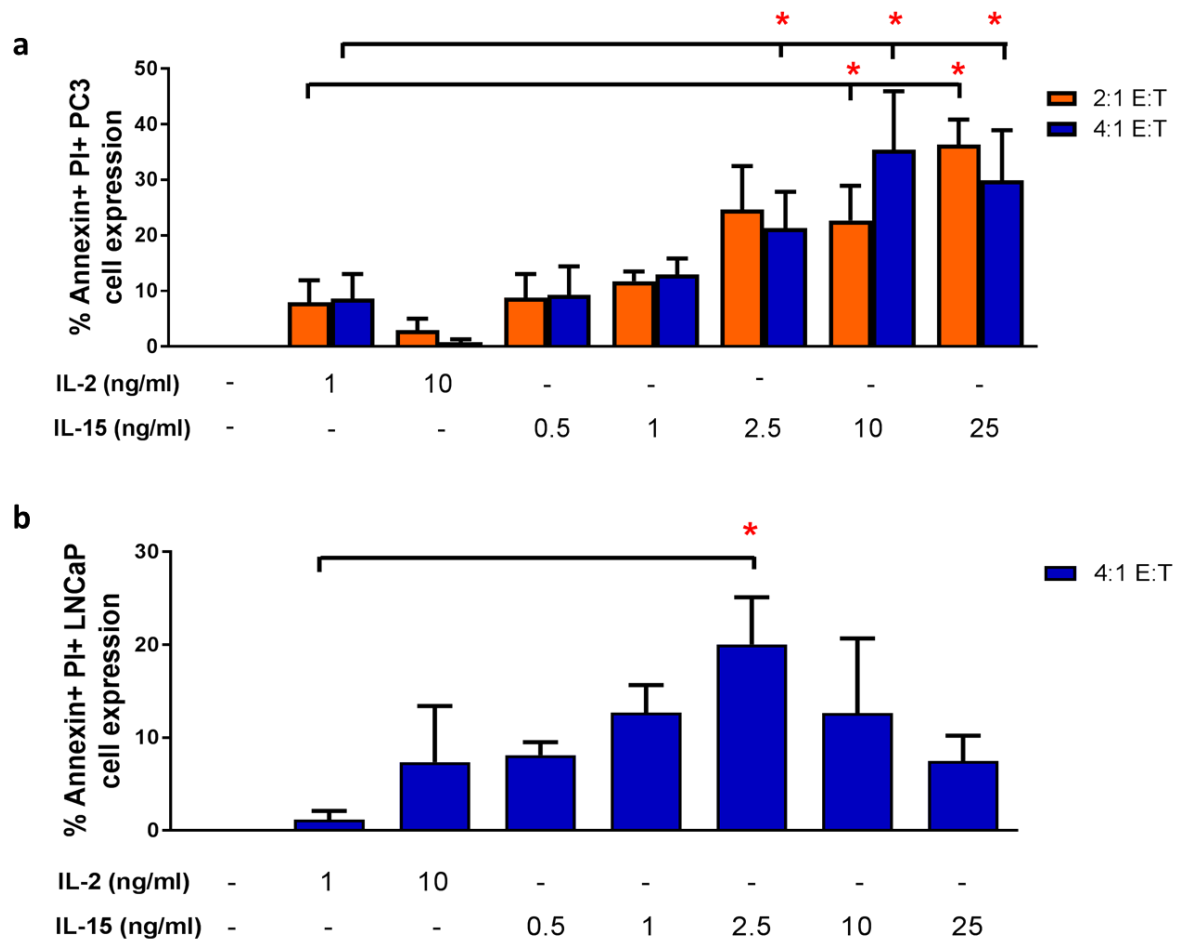


Figure 3.18: Annexin<sup>+</sup> PI<sup>+</sup> cell expression by PCa cells in varying coculture ratios of non-adherent PBMCs with PC-3 or LNCaP cells. **a)** Non- adherent PBMCs were cocultured at 2:1 and 4:1 E:T ratios with PC-3 cells, in the presence or absence of various concentrations of IL-2 and IL-15 **b)** Non- adherent PBMCs were cocultured at 4:1 E:T ratio with LNCaP cells, in the presence or absence of various concentrations of IL-2 and IL-15 (n=4, \*P<0.05, 1-way ANOVA).

The optimal ratio of immune effector cells to the target cells that would result in the highest percentage PCa cell death was found to be 4:1 for cocultures with both PC-3 and LNCaP cell lines and at an IL-15 concentration of 25ng/ml. The next step was to test the efficacy of the other Th1 immunotherapeutic cytokines on killing the PCa cells in comparison to control and IL-15. As above the addition of 600 $\mu$ M H<sub>2</sub>O<sub>2</sub> was used as a positive control. The percentage cell death was assessed by Annexin and PI double positive cell staining and the cell apoptosis and necrosis was measured using the same equation as above.

Non-adherent PBMCs led to the highest cytotoxicity towards PC-3 and LNCaP cell lines in the presence of IL-15 (figure 3.19 a, b) (n=4, \*P<0.05, 1-way ANOVA). In cocultures with PC-3 cells, the presence of IL-12 and of IL-21 also led to an increased cytotoxicity, however that was not significant when compared to the control or the other cytokines (n=4, p>0.05, 1-way ANOVA).

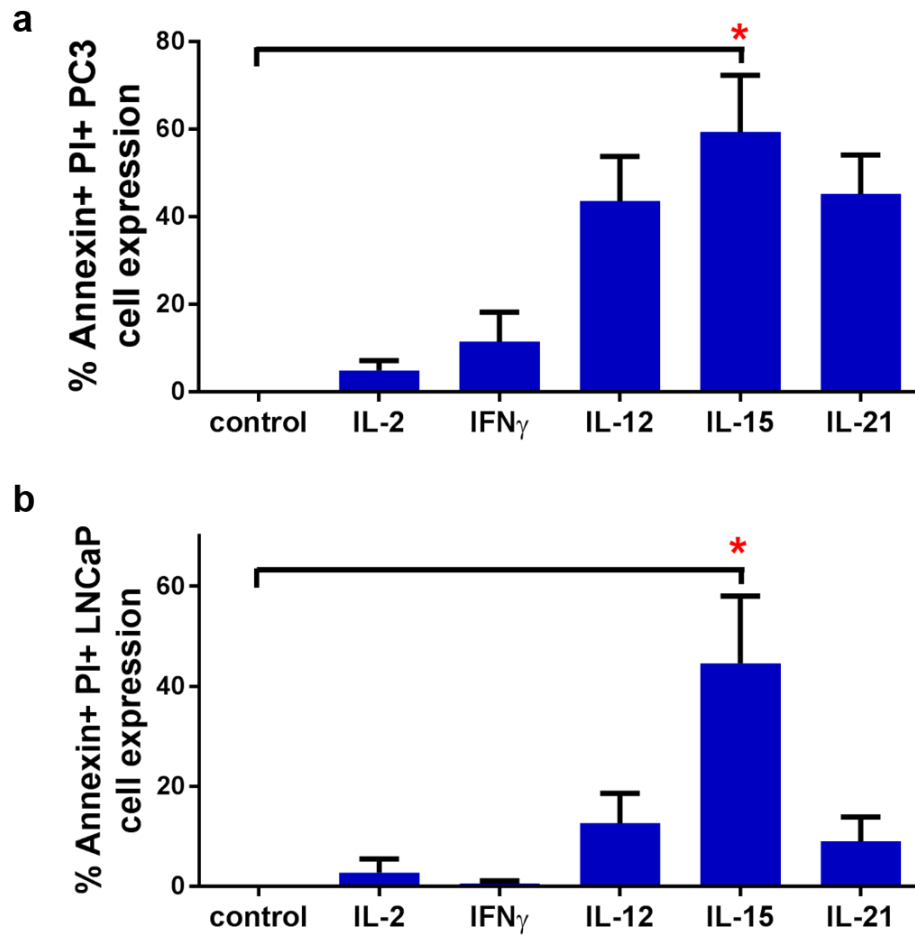


Figure 3.19: Annexin<sup>+</sup> PI<sup>+</sup> cell expression by PCa cells when non-adherent PBMCs were cocultured with PC-3 or LNCaP cells at 4:1 E:T ratio. Non-adherent PBMCs were cocultured at 4:1 E:T ratio with **a)** PC-3 cells or **b)** LNCaP cells, in the presence or absence of a panel of therapeutic Th1 cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21 (n=4, \*P<0.05, 1-way ANOVA).

### 3.2.3 Optimisation of the immune effector cell- target cell ratio on the syngeneic coculture model

Alloreactivity may be influencing the expansion of the NK and NKT in the above allogeneic coculture model, hence a syngeneic model was developed in order to investigate whether the effects seen above are due to alloreactivity. In this syngeneic system NK or T cells isolated from splenocytes of a murine C57BL/6 mouse were cocultured with the PCa cell line TRAMP-C1, also derived from a C57BL/6 mouse; in this set of experiments the first step was also to optimise the E:T ratio.

NK cells were isolated and cocultured at varying ratios with TRAMP-C1 cells in the presence or absence of 2.5ng/ml IL-15. The immune effector cell expansion (NK cells: NK1.1<sup>+</sup> CD3<sup>-</sup>; NKT cells: NK1.1<sup>+</sup> CD3<sup>+</sup>) and the TRAMP-C1 cell killing (PI<sup>+</sup> cells) by the immune effector cells was measured by flow cytometry.

The highest percentage of TRAMP-C1 cell death was observed at 1:1, 2:1 and 4:1 E:T ratios in the presence of IL-15, when compared to control (no cytokine) (n=5, \*p<0.05, 1-way ANOVA) (figure 3.20). IL-15 significantly expanded the NK cells compared to the control, as observed by NK1.1<sup>+</sup> CD3<sup>-</sup> cell expression, at the 4:1 E:T ratio (n=5, \*p<0.05, 1-way ANOVA) (figure 3.21), hence the ratio that was used on further experiments was 4:1.

The TRAMP-C1 cells were also cultured in the presence or absence of IL-15, in order to verify whether there is any direct effect of this cytokine on PCa cell death (Figure 3.22). The TRAMP-C1 cell death was not significantly affected by the presence of IL-15 when compared to the control (n=5, P>0.05, 1-way ANOVA).

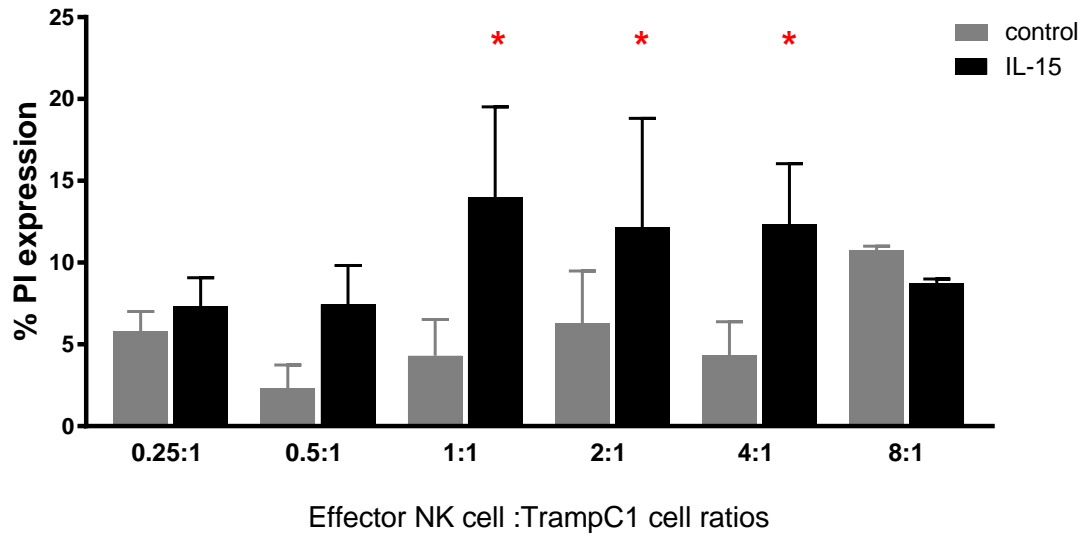


Figure 3.20: PI<sup>+</sup> cell expression by TRAMP-C1 cells when NK isolated cells were cocultured with TRAMP-C1 cells. NK isolated cells were cocultured with TRAMP-C1 cells at different E:T ratios, in the presence or absence of 2.5ng/ml IL-15. The TRAMP-C1 cell number was a constant number of  $1 \times 10^5$  cells, whereas the number of NK cells varied between  $25 \times 10^4$  cells -for the lowest ratio 0.25:1- and  $8 \times 10^5$  cells – for the highest ratio 8:1 (n=5, \* P<0.05, 1-way ANOVA).

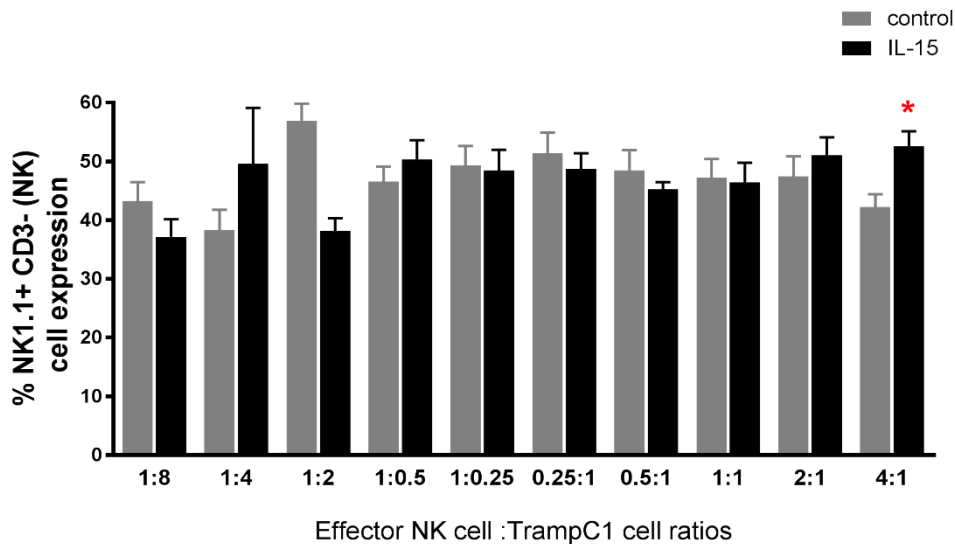


Figure 3.21: NK cell expansion at different coculture ratios of isolated NK cells and TRAMP-C1 cells. The expansion of NK cells is shown, as measured by NK1.1<sup>+</sup> CD3<sup>-</sup> cell expression; NK isolated cells were cocultured at different E:T ratios, in the presence or absence of 2.5ng/ml IL-15. The ratios that were used had variable E:T cell numbers, shown in the table 3.1 (n=5, \*P<0.05, 1-way ANOVA).

E:T ratio	4:1	2:1	1:8	1:4	1:2	1:1	1:0.5	1:0.25	0.5:1	0.25:1
Effector cell number	$4 \times 10^5$	$2 \times 10^5$	$1 \times 10^5$	$1 \times 10^5$	$1 \times 10^5$	$1 \times 10^5$	$1 \times 10^5$	$1 \times 10^5$	$5 \times 10^4$	$25 \times 10^4$
Target cell number	$1 \times 10^5$	$1 \times 10^5$	$8 \times 10^5$	$4 \times 10^5$	$2 \times 10^5$	$1 \times 10^5$	$5 \times 10^4$	$25 \times 10^4$	$1 \times 10^5$	$1 \times 10^5$

Table 3.1: Cell counts of effector and target cells used in each ratio of cocultures are shown.

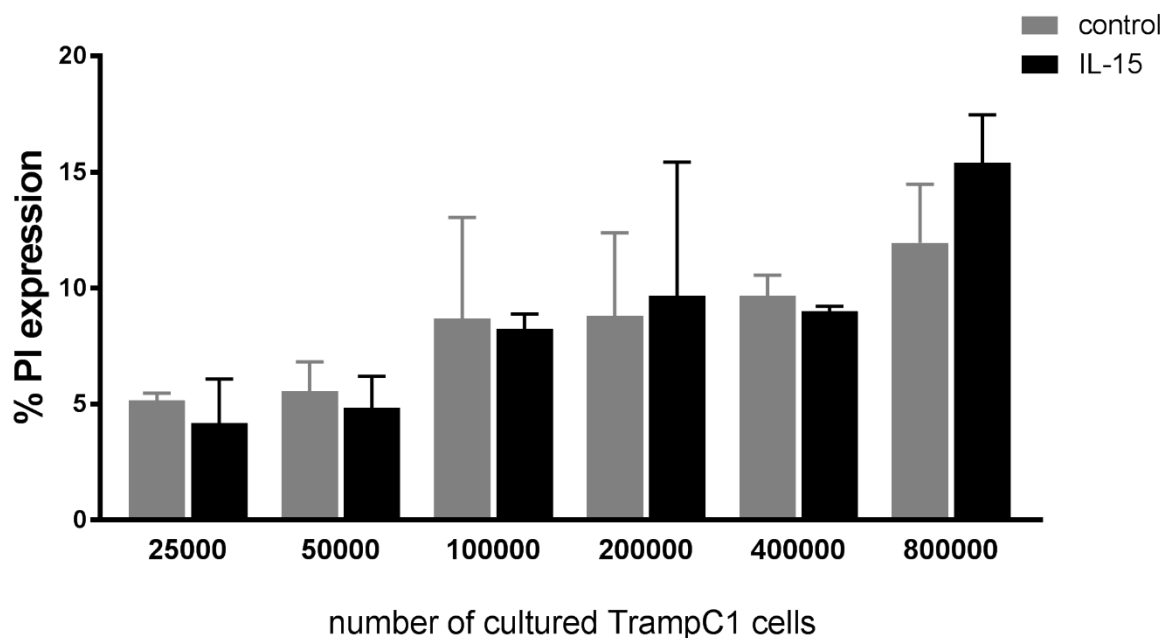


Figure 3.22: PI<sup>+</sup> cell expression by TRAMP-C1 cells, when they were cultured alone. TRAMP-C1 cells were cultured at varying cell concentrations in the presence or absence (control- no cytokine) of 2.5ng/ml IL-15 (n=5, P>0.05, 1-way ANOVA).



### 3.2.4 Screening of Th1 cytokines in the *in vitro* syngeneic immune effector cell – target cell coculture model

NK or T enriched cells from C57BL/6 mouse splenocytes were cocultured in the presence or absence of the TRAMP-C1 PCa cell line, derived from the C57BL/6 mouse prostate cancer, at a 4:1 E:T ratio, in the presence or absence of a panel of murine Th1 therapeutic cytokines (10ng/ml IL-2, 2.5ng/ml IFN $\gamma$ , 2.5ng/ml IL-12, 2.5ng/ml IL-15, 2.5ng/ml IL-21).

IL-15 is the only cytokine which highly expands the NK (NK1.1<sup>+</sup>CD3<sup>-</sup>) and NKT (NK1.1<sup>+</sup> CD3<sup>+</sup>) cells, compared to the control, IL-2, IFN $\gamma$ , IL-12 and IL-21 cytokines, when enriched NK cells from splenocytes are cultured alone (data not shown). Furthermore, in cocultures with TRAMP-C1 cells, IL-15 expands the NK and NKT cells even more, compared to when NK cells were cultured on their own and compared to control and the other cytokines. When T enriched cells were cocultured with TRAMP-C1 cells with or without cytokines, the presence of IL-15 highly expanded the CD8<sup>+</sup> CD4<sup>-</sup> T cells (Figure 3.23a-c) (n=5, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, 1-way ANOVA). T regulatory cell (CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup>) expression is not affected by IL-15, neither in the allogeneic coculture model as shown in the preliminary results (figure 3.4), nor in the syngeneic coculture system (Figure 3.24). Surprisingly, IL-2 does not have any effect on the Treg expression, when T enriched cells were cocultured with TRAMP-C1 cells (n=3, P>0.05, 1-way ANOVA).

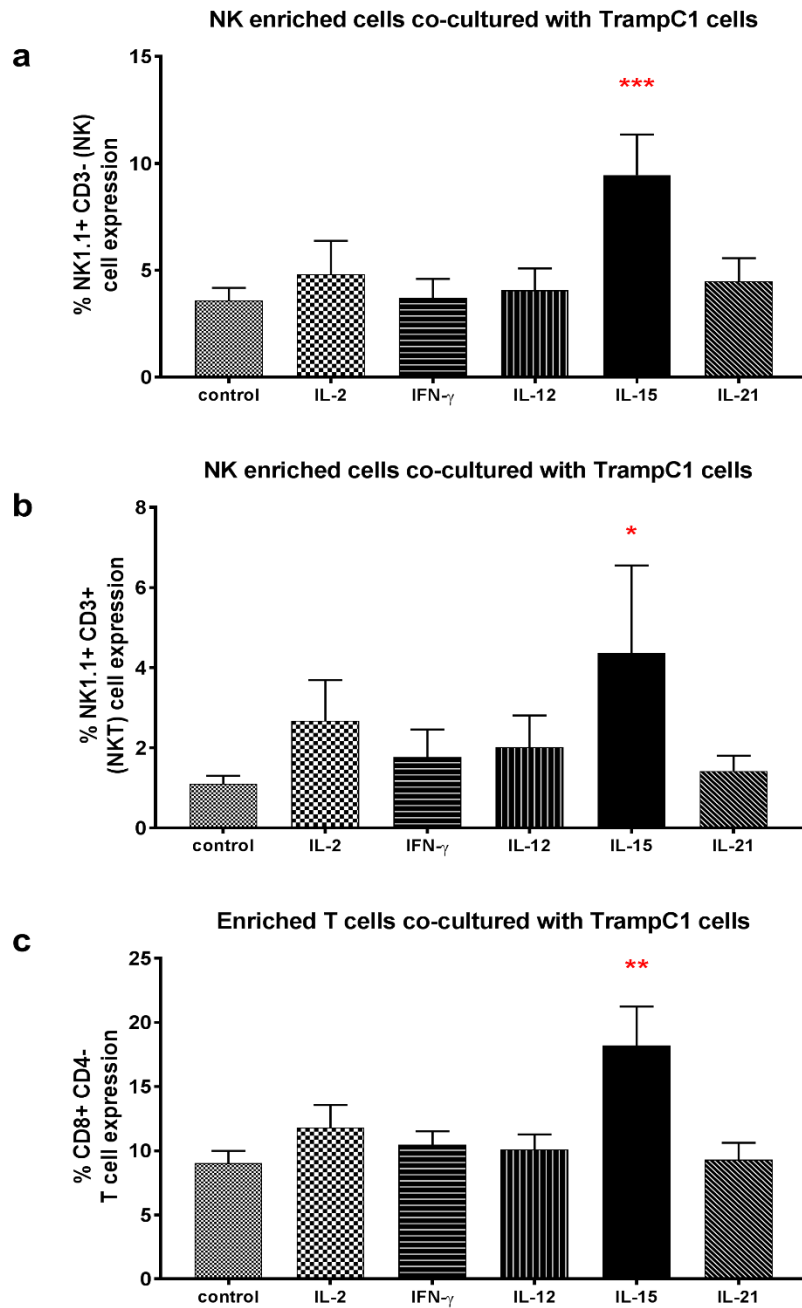


Figure 3.23: NK, NKT and CD8 T cell expansion when NK- or T- enriched cells were cocultured with TRAMP-C1 cells. The expansion of **a)** NK cells (NK1.1<sup>+</sup> CD3<sup>-</sup>), **b)** NKT cells (NK1.1<sup>+</sup> CD3<sup>+</sup>), **c)** CD8 T cells (CD8<sup>+</sup> CD4<sup>-</sup>) in the presence or absence of a panel of therapeutic cytokines (IL-2 10ng/ml; 2.5ng/ml IFN $\gamma$ ; 2.5ng/ml IL-12; 2.5ng/ml IL-15; 2.5ng/ml IL-21) is shown, when NK-enriched or T- enriched cells were cultured in the presence of TRAMP-C1 cell line at 4:1 E:T ratio (n=5, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, 1-way ANOVA).

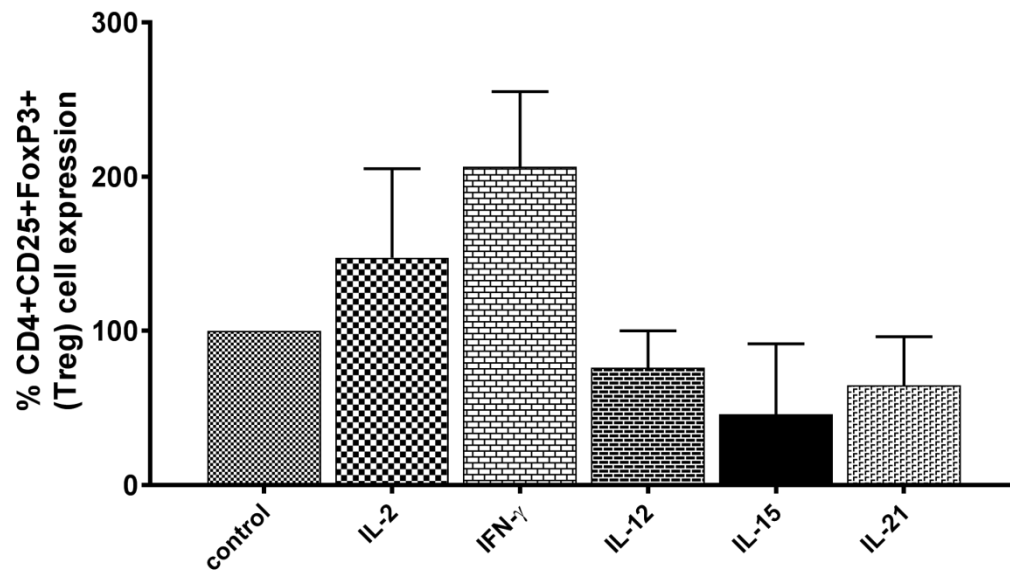


Figure 3.24: Treg cell expansion when T-enriched cells were cocultured with TRAMP-C1 cells. The expansion of Treg cells is shown, when T enriched cells were cocultured with the TRAMP-C1 cell line, in the presence or absence of a panel of therapeutic Th1 cytokines (10ng/ml IL-2, 2.5ng/ml IFN $\gamma$  , 2.5ng/ml IL-12, 2.5ng/ml IL-15, 2.5ng/ml IL-21) (n=3, P>0.05, 1-way ANOVA).

### **3.3 Discussion**

An *in vitro* allogeneic coculture model of immune effector cells and PCa cell lines was established in our laboratory. This coculture model mimics the PCa microenvironment and it has enabled us to study the effects of a panel of therapeutic Th1 cytokines (e.g. IL-2, IL-12, IL-15) on the immune effector cell proliferation and activity.

In preliminary data from our lab we have established an allogeneic model in which non-adherent PBMCs were cocultured in the presence or absence of either PC-3 or LNCaP cell lines in the presence or absence of a panel of therapeutic Th1 cytokines. It was found that IL-15 is the only cytokine that increases significantly the expansion of NK, NKT and CD8 T cells when compared to control (no cytokine) or to the other cytokines and when non-adherent PBMCs were cultured alone. The effects of IL-15, aiding the function and expansion of NK cells have been previously studied and established in literature (Carson, Giri et al. 1994, Huntington, Legrand et al. 2009) . However, in our coculture model, it was observed that in the presence of the PCa cell lines, IL-15 increased the expansion of the immune effectors cells, NK, NKT and CD8 T cells to a greater extent than when lymphocytes are cultured alone. Additionally, IL-15 aided the killing of PCa cells by the immune effector cells, unlike any other cytokine and this was observed both from an annexin and PI surface staining on the PCa cell lines, as well as from the upregulation of markers of cytotoxicity (e.g. Perforin, CD107a) (Elhage, Galustian et al. 2011).

The first aim of this chapter was to verify whether the effector to target (E:T) coculture ratio that was used in the preliminary experiments, 8:1, was optimal for the greatest expansion of NK, NKT and CD8 T cells, with a concomitant enhancement of the PCa cytotoxicity by these immune effector cells, as various E:T ratios have been used in literature previously (Marten, Renoth et al. 2001, Balsamo, Vermi et al. 2012).

A panel of varying coculture E:T ratios were investigated for the best expansion of immune effector cells and their cytotoxicity towards the PCa cell lines. It was found that the highest cell killing by these immune effector cells towards the PC-3 cell line was induced at a 4:1 or 2:1 ratio and in the presence of 2.5ng/ml of IL-15; in the cocultures with the LNCaP cell line,

the highest percentage cell killing as measured by Annexin and PI (markers for apoptosis and necrosis respectively) surface staining, was observed at a 4:1 ratio and 2.5ng/ml of IL-15.

At the 8:1, 4:1 and 2:1 ratios, the presence of IL-15- at a concentration of 2.5ng/ml- highly expanded the NK and NKT cells, their perforin and CD107a expression in cocultures with both PCa cell lines. Taking these results together and since the highest percentage killing of LNCaP cells in the presence of IL-15, was produced at a 4:1 E:T ratio only, it was decided that the optimal E:T coculture ratio for the allogeneic system is 4:1 and this would be used in further experiments.

When non-adherent PBMCs were cultured on their own, the presence of IL-15 highly expanded NK, NKT and CD8 T cells, as shown in the preliminary results as well. However it was shown that in the presence of PCa cells, the IL-15 mediated expansion of NK and NKT cells was even higher.

CD8 T cells did not seem to significantly expand in the cocultures of non-adherent PBMCs with PCa cells in the presence of IL-15, when compared to control; this result is not in agreement with the preliminary data shown in figure 3.2. However, the concentration of IL-15 that was used in the preliminary experiment was 25ng/ml, whereas in further experiments that were done 2.5ng/ml of IL-15 were used, a concentration which was shown to induce the maximum NK cell expansion (figure 3.5).

The coculture model that was used for the preliminary work and for the optimisation experiments was an allogeneic coculture model (ie. Peripheral blood mononuclear cells are not derived from the same donor as the PCa cells), so it could be argued that alloreactivity may be promoting expansion of the NK and NKT cells in the presence of IL-15. Therefore, another aim of this study, was to create a syngeneic murine *in vitro* coculture model, in which murine NK or T cells purified from splenocytes of C57BL/6 mice, were cocultured with the TRAMP-C1 cell line, a PCa cell line also derived from transgenic adenocarcinoma of the C57BL/6 mouse prostate.

The presence of IL-15 in the cocultures, produced a highly significant increase in the killing of the TRAMP-C1 cells by the NK cells, as observed by the PI<sup>+</sup> cell expression, compared to the control (no cytokine), at E:T ratios of 1:1, 2:1 and 4:1. However the most significant increase

in the expansion of NK1.1<sup>+</sup> NK cells, was observed at an E:T ratio of 4:1. Based on this, it was decided that the optimal ratio that would be used for further experiments was 4:1.

Taking into account the observations so far, it is understood that the presence of IL-15 in cocultures of immune effector cells with PCa cell lines, enhances the expansion of the immune effector cells and their cytotoxic activity towards the PCa cell lines. However, the direct effect of IL-15 on the PCa cell lines, was not investigated; therefore in order to examine whether IL-15 plays any role on the TRAMP-C1 cell death, TRAMP-C1 cells were cultured alone, in the presence or absence (control) of IL-15 and at different cell concentrations. As seen in figure 3.22, 5-15% of TRAMP-C1 cells seem to be necrotic and the percentage cell death is proportional to the amount of cells that are added per well; the higher the cell concentration in a well, the higher the cell death. Nevertheless, the presence of IL-15 does not seem to have any effect on the TRAMP-C1 cell death, when compared to the control, hence it is concluded that IL-15 is only affecting and modulating the NK and the T cell function in the coculture models. Unfortunately, a drawback in the cell killing experiments on the syngeneic coculture model, was the absence of a positive control; on the allogeneic coculture models H<sub>2</sub>O<sub>2</sub> was added on the PCa cell lines (PC-3 and LNCaP), to observe the maximum percentage necrosis of the PCa cells. In future, these experiments should be repeated with the addition of a positive control.

In order to investigate whether a panel of Th1 therapeutic cytokines, that were also used in our allogeneic models, had any effect on the expansion and activity of NK and T purified cells in the syngeneic model, we cocultured NK enriched cells and T enriched cells in the presence or absence of TRAMP-C1 cells, and in the presence or absence of each of the cytokines. It was observed that IL-15 is the most potent activator of NK, NKT and CD8 T cells either in the presence or absence of TRAMP-C1 cells, when compared to the control or to the other Th1 cytokines. These results are in agreement with those from the allogeneic coculture model, meaning that alloreactivity does not have any effect on the results we are observing. Unfortunately, the perforin and CD107a expression by the NK, NKT and CD8 T cells was not examined in the syngeneic coculture system, which should also be done in the future, in order to observe whether the presence of IL-15 in cocultures also has an effect on the markers of cytotoxicity of the immune effector cells.

To conclude, IL-15 has been previously shown to enhance NK and T cell proliferation (Fehniger, Suzuki et al. 2001) (Gravisaco, Mongini et al. 2003, Sato, Sabzevari et al. 2011) . However, to our knowledge we are the first group in the UK, to show that IL-15 is also active when effector immune cells are cultured in the presence of PCa cell lines.

## CHAPTER 4

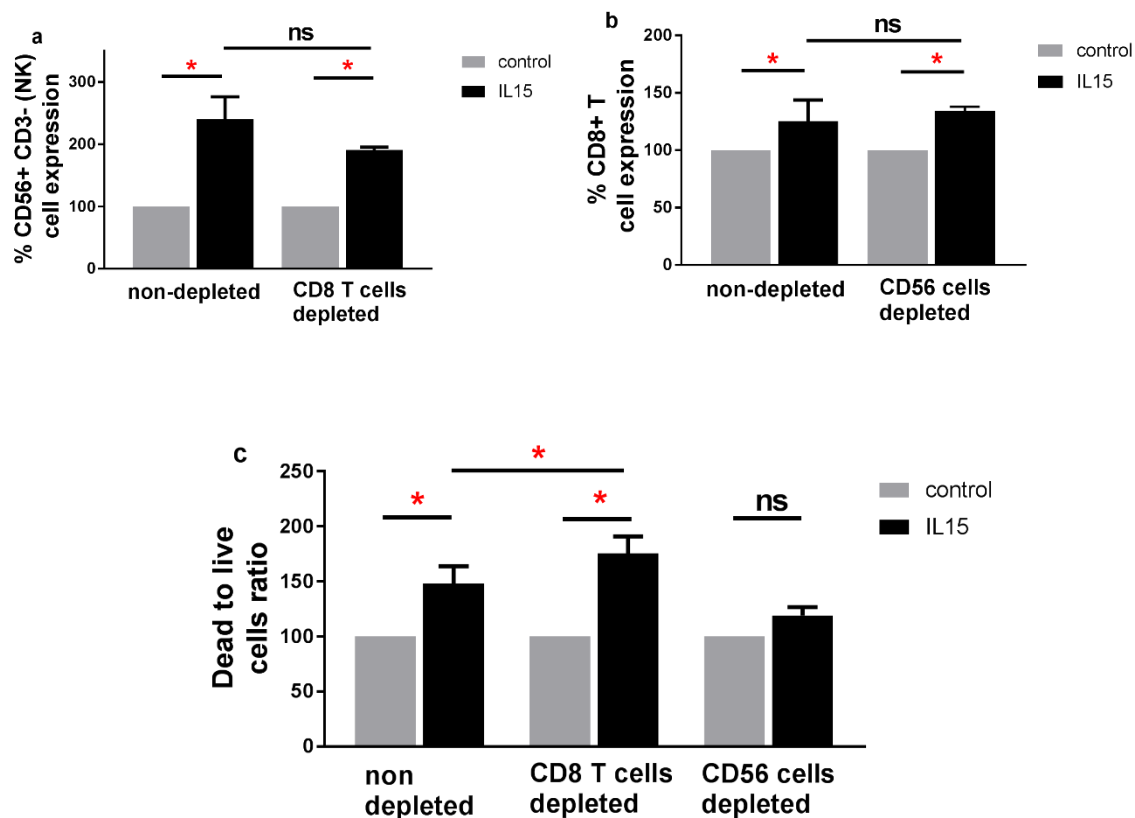
Studies of the mechanism of action of IL-15 on NK and NKT cells-  
modulation of NK receptors and ligands on NK cells in the allogeneic  
coculture model



## 4.1 Introduction

In parallel to this work, Mr Oussama Elhage from the Protein Therapeutics Laboratory, investigated whether the enhanced cytotoxicity of the non-adherent PBMCs towards the PCa cell lines in the presence of IL-15, was attributed to NK or CD8 T cells. He did that by depleting CD56<sup>+</sup> cells (NK and NKT cells) and CD8<sup>+</sup> T cells in the allogeneic coculture model, and then examining the IL-15 mediated effector cell induced PCa cell killing and expansion of CD8<sup>+</sup> and CD56<sup>+</sup> cells respectively, within the cocultures.

It was observed that the depletion of CD8 T cells does not affect the CD56<sup>+</sup> (NK) cell expansion and that CD56<sup>+</sup> cell depletion does not affect the CD8 T cell expansion, when compared to the non-depleted cells, both in the presence and absence of IL-15 (n=5, \*p<0.05, 1-way ANOVA) (Figure 4.1a,b). Additionally, it was observed that when CD8 T cells were depleted, the presence of IL-15 enhanced the CD56<sup>+</sup> (NK) cytotoxicity towards the PC-3 cells, as observed by the dead to live PC-3 cells ratio, when compared to the control (n=5, \*p<0.05, 1-way ANOVA) (Figure 4.1c). When CD56<sup>+</sup> cells were depleted, the percentage of dead to live PC-3 cells, was similar in the control and in the presence of IL-15, meaning that IL-15 dependent PC-3 cell cytotoxicity may be attributed to the NK cells and not the CD8 T cells. The NKT cells' effect was not examined, hence it cannot be excluded that NKT cells also play a role in the IL-15 mediated cytotoxicity.



**Figure 4.1: CD56 and CD8 cell depletions: expansion and cell killing assay** **a)** Expansion of NK cells as measured by the CD56<sup>+</sup> CD3<sup>-</sup> expression, when non-adherent PBMCs or CD8 T-depleted cells were cocultured with PC-3 cells in the presence or absence of IL-15. **b)** Expansion of CD8<sup>+</sup> T cells, when non-adherent PBMCs or CD56<sup>+</sup> depleted cells were cocultured with PC-3 cells, in the presence or absence of IL-15 (n=5, \*p<0.05, 1-way ANOVA). **c)** Dead to live PC-3 cells ratio shown, when non-adherent PBMCs, CD8 T-depleted cells or CD56 - depleted cells were cocultured with PC-3 cells in the presence or absence (control) of IL-15 (n=5, \*p<0.05, 1-way ANOVA).

NK cells have the ability to distinguish transformed cells from healthy cells, via the receptors that they bear on their surface. These are either inhibitory (iNKRs) or activatory receptors (aNKRs) that regulate the killing of the target cells; the iNKRs recognise molecules of the Major Histocompatibility Complex-I (MHC-I) and in humans these are called Killer Immunoglobulin- like Receptors (KIR). Tumour cells express low levels of MHC-I molecules, hence they often escape the NK cell recognition through this inhibitory signalling (Bottino, Castriconi et al. 2005). In order for NK cells to be able to distinguish the cancer cells, they rely on stimulatory signals that are delivered via the aNKRs; these directly trigger the NK cell to kill

its target by the release of cytokines (e.g. IFN $\gamma$ ) and through the release of cytotoxic granules, which are stored within secretory lysosomes (Topham and Hewitt 2009). (More info on NKRs can be found on sections 1.2.2.1 and 1.2.3 in this thesis).

Following these observations, we wanted to determine whether IL-15 was modulating the expression of activatory or inhibitory NK receptors (or their ligands) on NK cells. IL-15 has been previously shown to have an effect on the expression of the activatory NKR NKG2D on NK cells (Sutherland, Chalupny et al. 2002, Muntasell, Magri et al. 2010), but its effects on this and other receptors have not been studied when effector cells are cultured with tumour cell lines, and particularly, PCa cell lines.

The aim of this part of the project was to investigate the mechanism/s by which IL-15 acts on effector cell mediated killing of PCa cells in the allogeneic coculture model, focusing on IL-15-mediated effects on NK cells. The NKRs and their corresponding ligands were studied in detail, in order to investigate whether IL-15 modulates the expression and shedding of these ligands.

A panel of various receptors was assessed in the presence or absence of IL-2 and IL-15 cytokines, in our *in vitro* allogeneic coculture model of non-adherent PBMCs and PC-3 or LNCaP cell lines. The ratio of cells that was used, was 4:1 PBMCs:PCa cells, as optimised on the previous chapter. The NKRs chosen for investigation have been found to play roles in immunosurveillance in previous literature (discussed further on sections 1.2.2.1 and 1.2.3) and antibodies to these were commercially available at the time that these experiments were done. The iNKRs that were studied were the Killer Immunoglobulin-like receptors (KIRs) KIR2DL1, KIR3DL1 and KIR2DL2. The aNKRs that were studied were: NKG2D, NKp30, NKp44, NKp46 and DNAM-1.

## **4.2 Results**

### **4.2.1 Effects of IL-2 and IL-15 cytokines on KIR family and on activatory receptor expression by NK cells**

In order to investigate the effects of IL-2 and IL-15 on both the inhibitory and activatory NKRs, non-adherent PBMCs were cultured either alone, or in the presence of low or high dose of IL-2, or in the presence of IL-15 or IL-15 and a low dose of IL-2. The low dose of IL-2 (1ng/ml) is normally found in human physiological processes, whereas the high dose (10ng/ml) could enhance the anti-tumour activity, as high doses of IL-2 are currently used in the treatment of melanoma and RCC as described in section 1.4.

In the presence of IL-15, the expression of the iNKR KIR2DL2 by the NK cells was highly upregulated, unlike in the presence of IL-2, where none of the receptors' expression was affected (n=12, \*\*p<0.01, 1-way ANOVA) (Figure 4.2a-c). In addition, in the presence of IL-15 the expression of the natural cytotoxicity receptor (NCRs) NKp46 was significantly upregulated (n=12, \*p<0.05, 1-way ANOVA) (Figure 4.3 d-f) as well as that of the aNKR DNAM-1 (n=8-11, \*p<0.05, 1-way ANOVA) (figure 4.4 g-h).

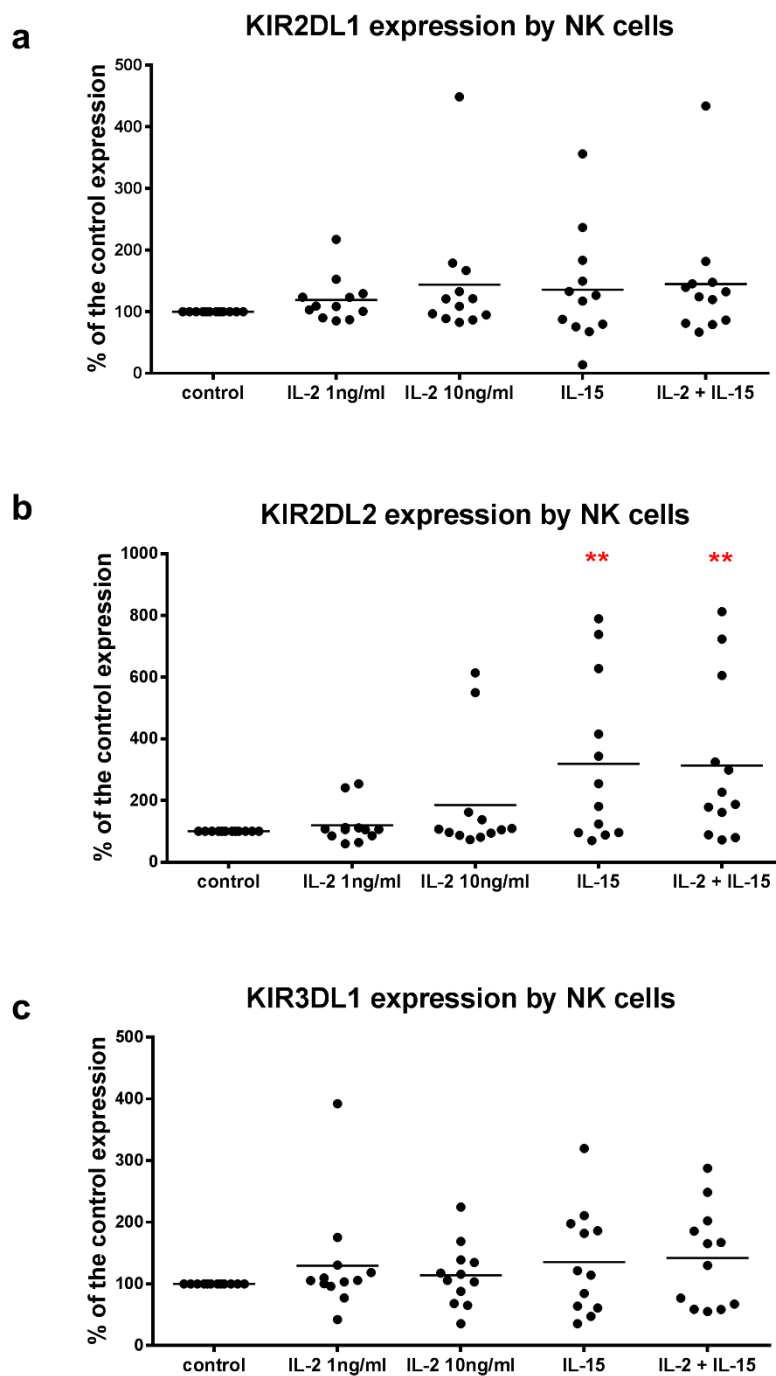


Figure 4.2 a-c: Expression of KIR2DL1, KIR2DL2 and KIR3DL1 by NK cells. Non-adherent PBMCs were cultured in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=12, \*\*p<0.01, 1-way ANOVA).

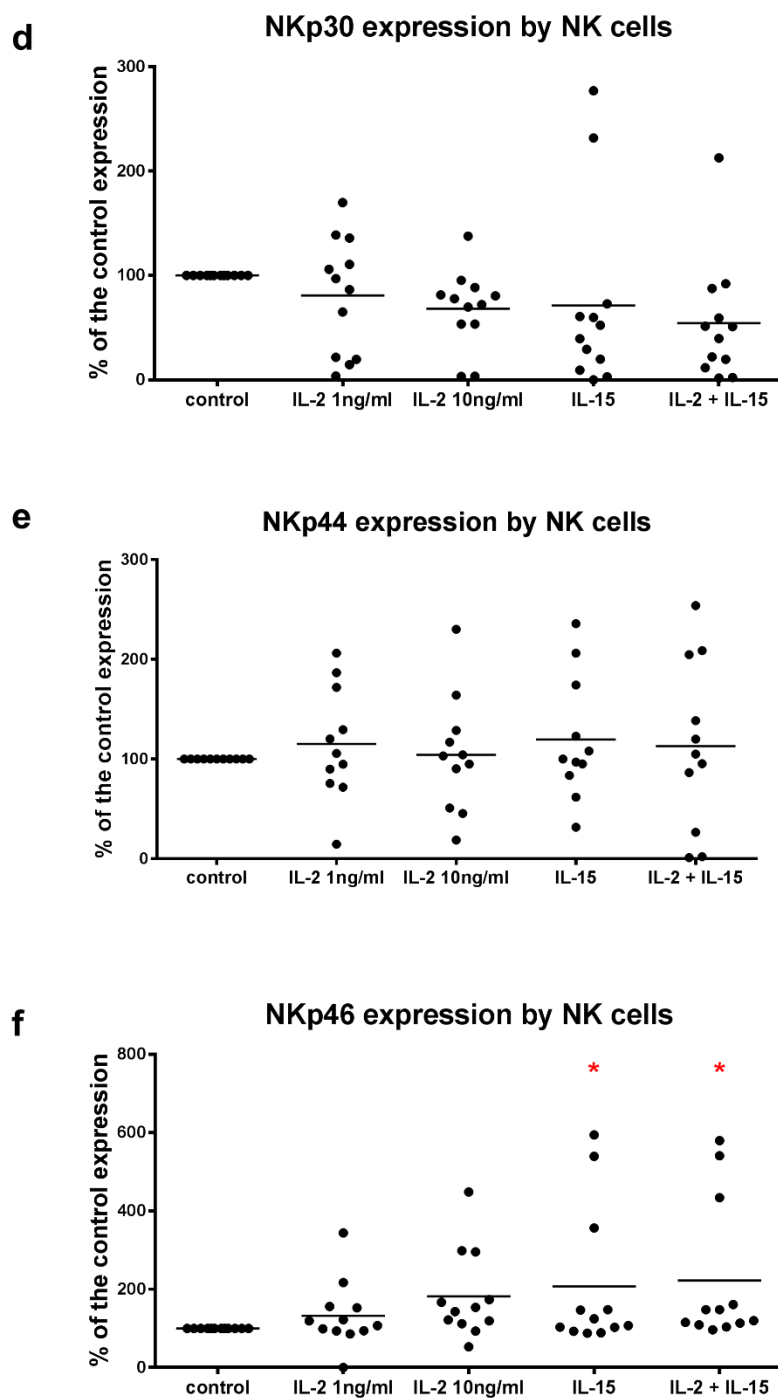


Figure 4.3 d-f: Expression of NCRs – NKp30, NKp44, NKp46 - by NK cells. Non-adherent PBMCs were cultured in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=12, \*p<0.05, 1-way ANOVA).

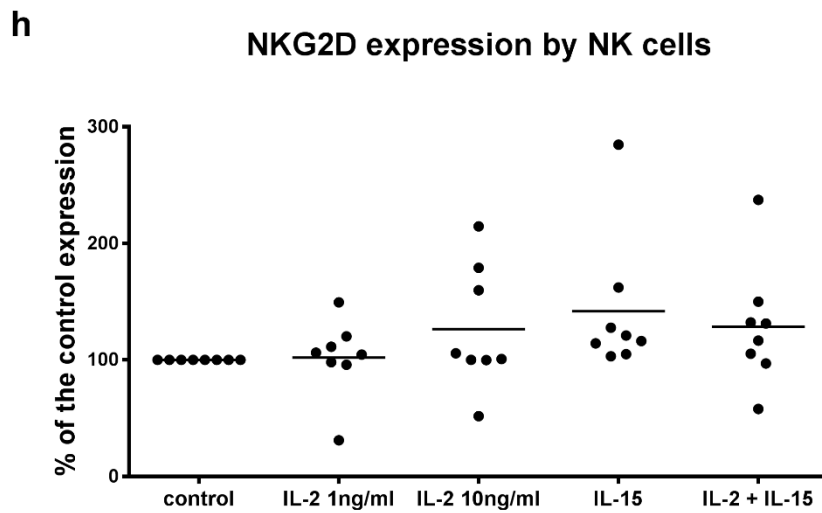
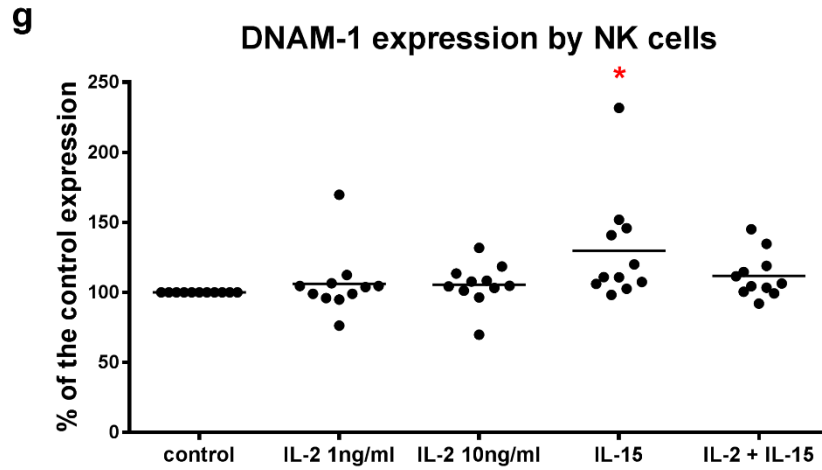


Figure 4.4 g,h: Expression of DNAM-1 and NKG2D by NK cells. Non-adherent PBMCs were cultured in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=8-11, \*p<0.05, 1-way ANOVA).

#### 4.2.2 Effects of IL-2 and IL-15 cytokines on the KIR family and on activatory receptor expression by NK cells in the coculture models of non-adherent PBMCs and PC-3 or LNCaP cell lines.

The next aim was to investigate the effects of IL-2 and IL-15 on both the inhibitory and activatory NKRs when non-adherent PBMCs were cocultured with PC-3 or LNCaP cell lines, either alone, or in the presence of low or high dose of IL-2, or in the presence of IL-15 or IL-15 and a low dose of IL-2.

For all the coculture model experiments, the E:T ratio used was 4:1, as optimised on chapter 3.

In the cocultures of non-adherent PBMCs with PC-3 cells, in the presence of IL-15 the expression of NKG2D aNKR was significantly upregulated when compared to control and IL-2 (Figure 4.5-4.7a-h) (n=7, \*p<0.05, 1-way ANOVA). The presence of IL-2 or IL-15 did not affect any of the other receptors' expression.



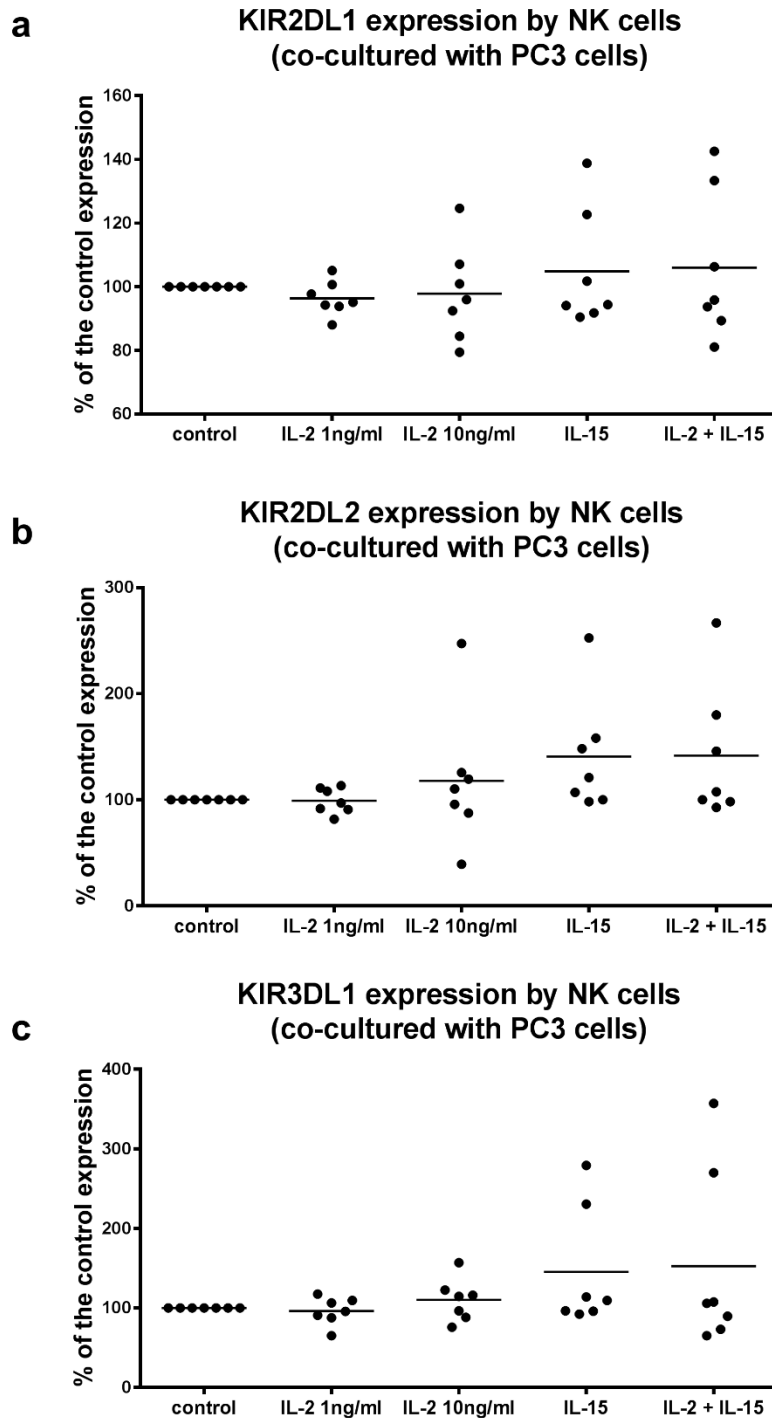


Figure 4.5 a-c: Expression of KIR2DL1, KIR2DL2 and KIR3DL1 by NK cells in cocultures with PC-3 cells. Non-adherent PBMCs were cocultured with PC-3 cells in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=5,  $p>0.05$ , 1-way ANOVA).

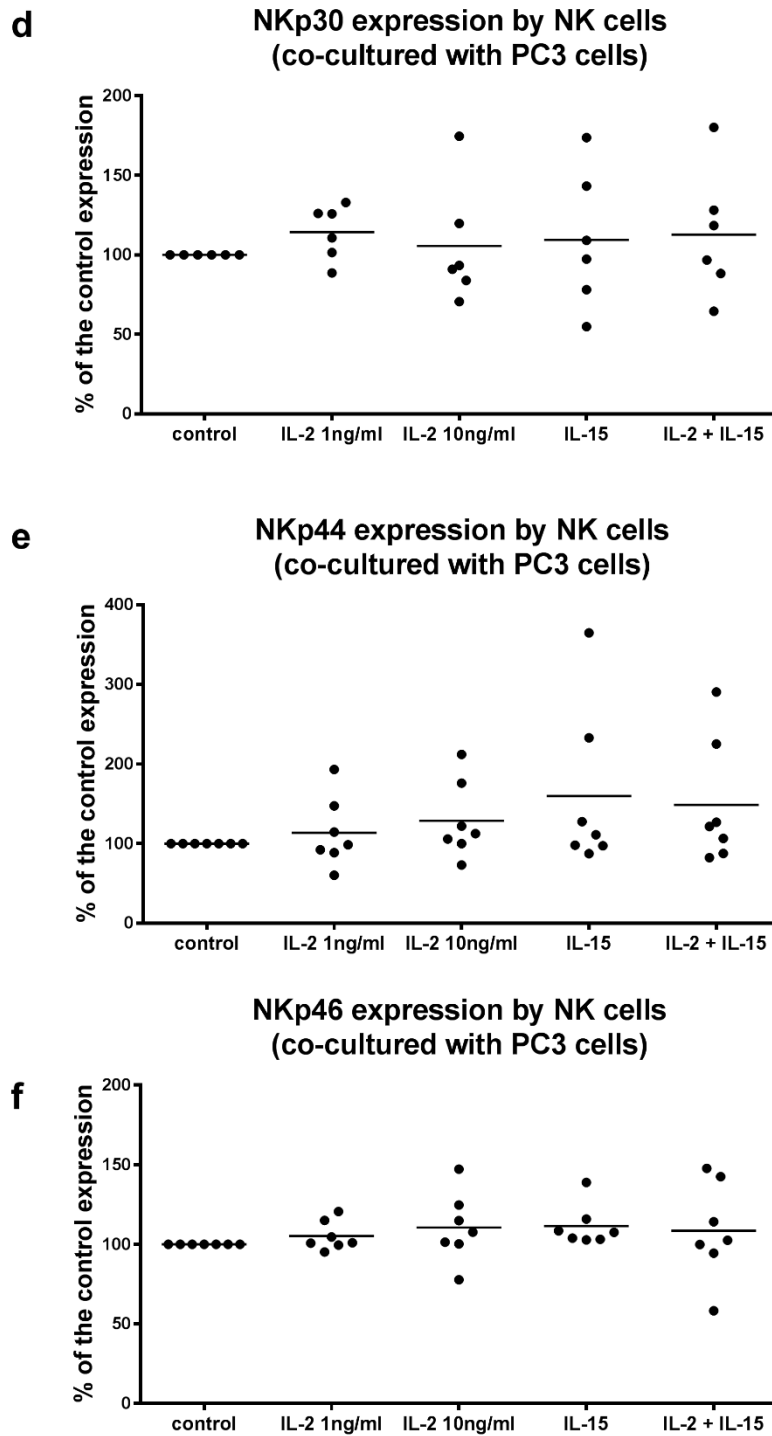


Figure 4.6 d-f: Expression of NCRs – NKp30, NKp44, NKp46 - by NK cells in cocultures with PC-3 cells. Non-adherent PBMCs were cocultured with PC-3 cells in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=7, p>0.05, 1-way ANOVA).

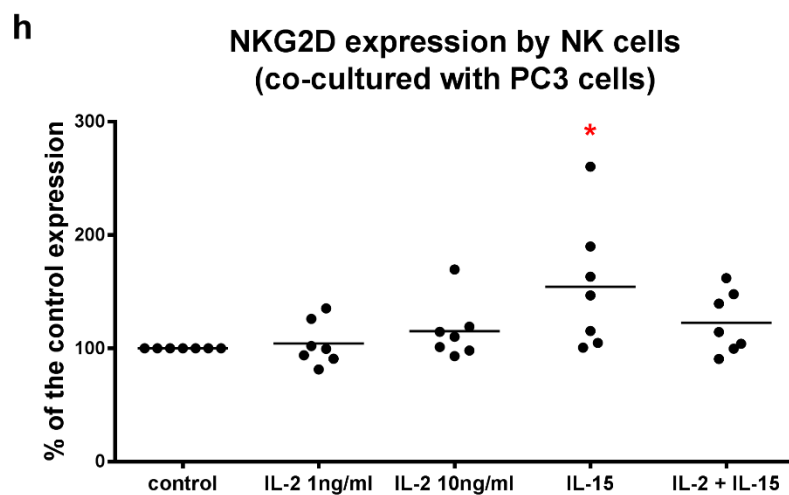
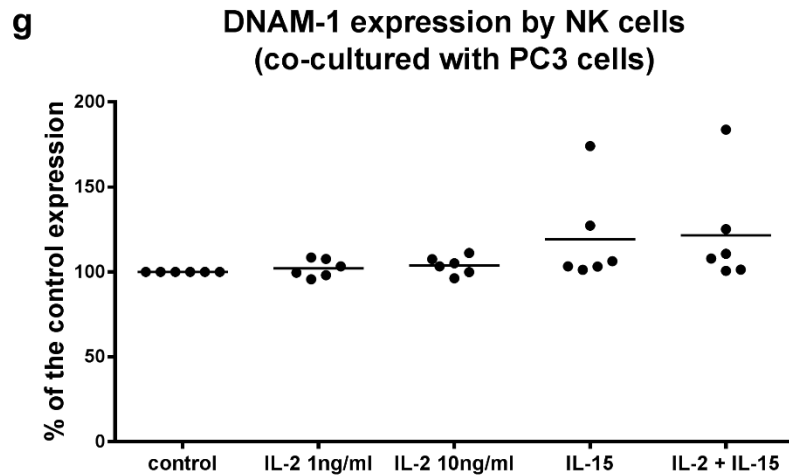


Figure 4.7 g,h: Expression of DNAM-1 and NKG2D by NK cells in cocultures with PC-3 cells. Non-adherent PBMCs were cocultured with PC-3 cells in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=7, \*p<0.05, 1-way ANOVA).

In the cocultures of non-adherent PBMCs with LNCaP cells (figures 4.8- 4.10), in the presence of IL-2 and of IL-15 the expression of DNAM-1 was upregulated. The presence of IL-15 however, additionally upregulated the expression of KIR2DL2 and downregulated the NKp30 expression (n=5, \*p<0.05, \*\*p<0.01, 1-way ANOVA).

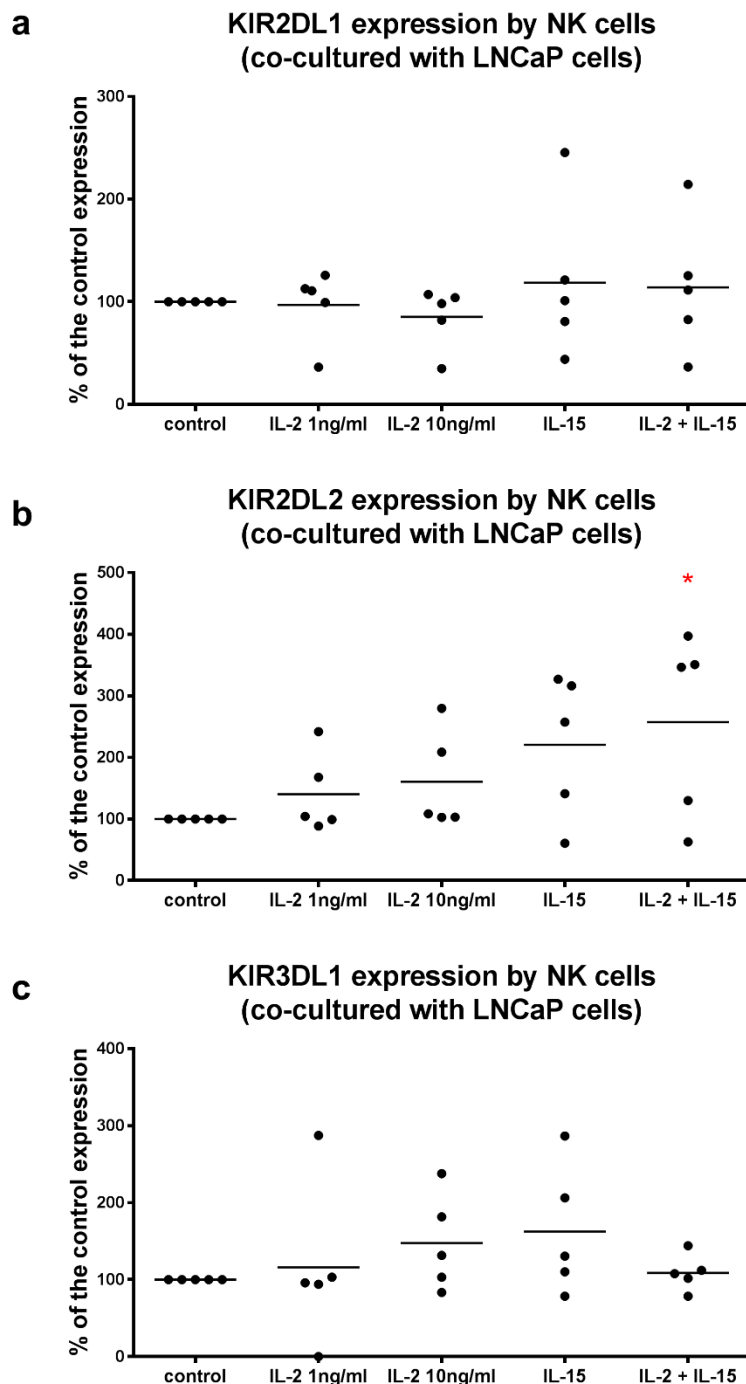


Figure 4.8 a-c: Expression of KIR2DL1, KIR2DL2 and KIR3DL1 by NK cells in cocultures with LNCaP cells. Non-adherent PBMCs were cocultured with LNCaP cells in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=5, p>0.05, 1-way ANOVA).

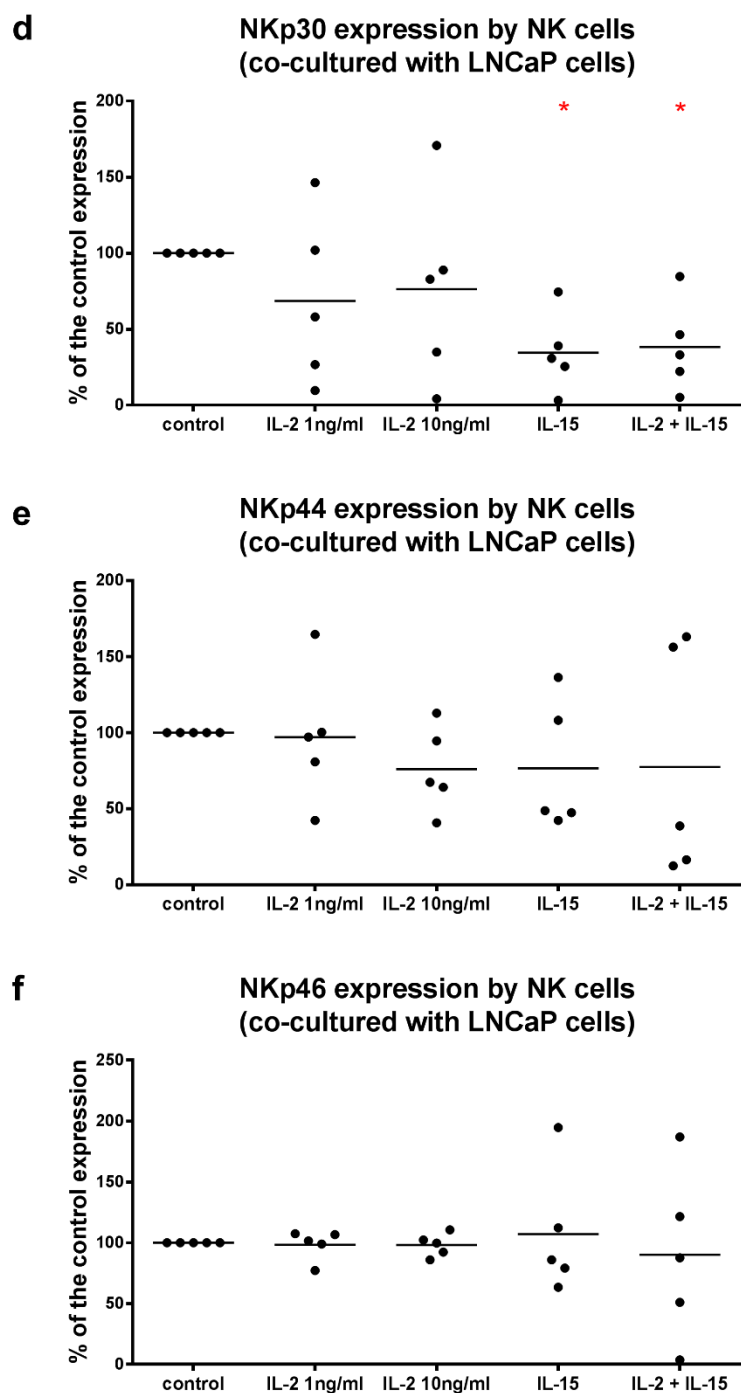


Figure 4.9 d-f: Expression of NCRs – NKp30, NKp44, NKp46 - by NK cells in cocultures with LNCaP cells. Non-adherent PBMCs were cocultured with LNCaP cells in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=5, \*p<0.05, 1-way ANOVA).

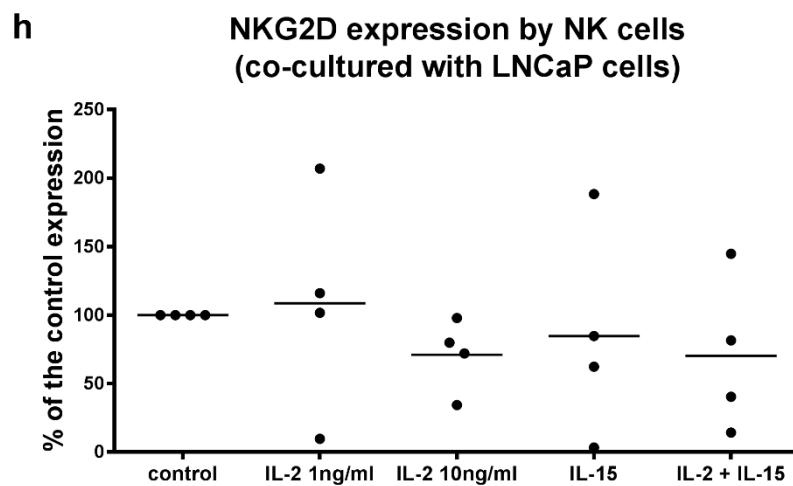
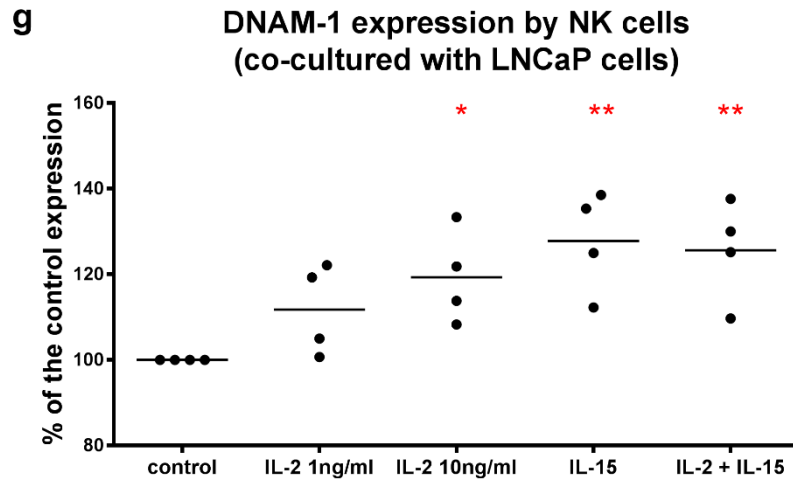


Figure 4.10 g,h: Expression of DNAM-1 and NKG2D by NK cells in cocultures with LNCaP cells. Non-adherent PBMCs were cocultured with LNCaP cells in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=4, \*p<0.05 \*\*p<0.01, 1-way ANOVA).

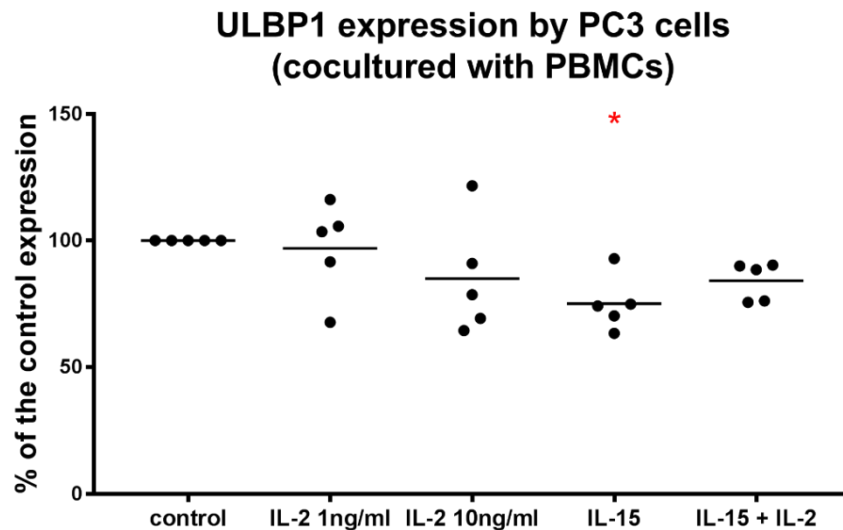
#### 4.2.3 Effects of IL-2 and IL-15 cytokines on ligand expression by the PCa cell lines, PC-3 and LNCaP cultured alone and in the allogeneic coculture model

A panel of NK cell receptor ligands expressed by PCa cells corresponding to the NK cell receptors was investigated in this study. The ligands that were studied included those of the NKG2D aNKR, MICA, MICA/B and ULBP1, the ligand Nectin-2 which is a ligand for the aNKR DNAM-1, the HLA-A,B,C ligand, recognised by T cell receptor interactions and lastly HLA-I Bw4, which is recognised by the KIR3DL1 iNKR.

For some of the receptors that were investigated in section 4.2.2-4.2.3 their ligands are unknown or antibodies were not commercially available, hence the ones described here were studied.

In order to investigate the expression of the ligands by the PCa cell lines, PC-3 and LNCaP cells were cultured on their own, in the absence of any cytokine or in the presence of low (1ng/ml) or high dose (10ng/ml) of IL-2 or in the presence of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml). No significant difference was observed in any of the ligands expressed by the PCa cell lines, in the presence of IL-15 and/or IL-2 cytokines, when compared to the control (no cytokine addition) (results shown in Appendix).

When the ligand expression by PC-3 cells was investigated in the cocultures with non-adherent PBMCs, a significant downregulation of the ULBP1 NK ligand was observed, in the presence of IL-15 (n=4, \*p<0.05, 1-way ANOVA) (Figure 4.11).



**Figure 4.11: Expression of ULBP1 by PC-3 cells in cocultures with PBMCs.** PC-3 cells were cocultured with non-adherent PBMCs cells in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=4, \*p<0.05, 1-way ANOVA).



When investigating the ligand expression by the LNCaP cell line when coculturing with non-adherent PBMCs in the presence or absence of IL-2 and IL-15 cytokines, it was observed that the presence of IL-15 significantly downregulated the expression of the ligands MICA, ULBP1, Nectin-2 and HLA-I Bw4 ( $n=4$ ,  $*p<0.05$ ,  $**p<0.01$ ,  $***p<0.001$ ,  $****p<0.0001$ , 1-way ANOVA) (Figures 4.12 and 4.13).

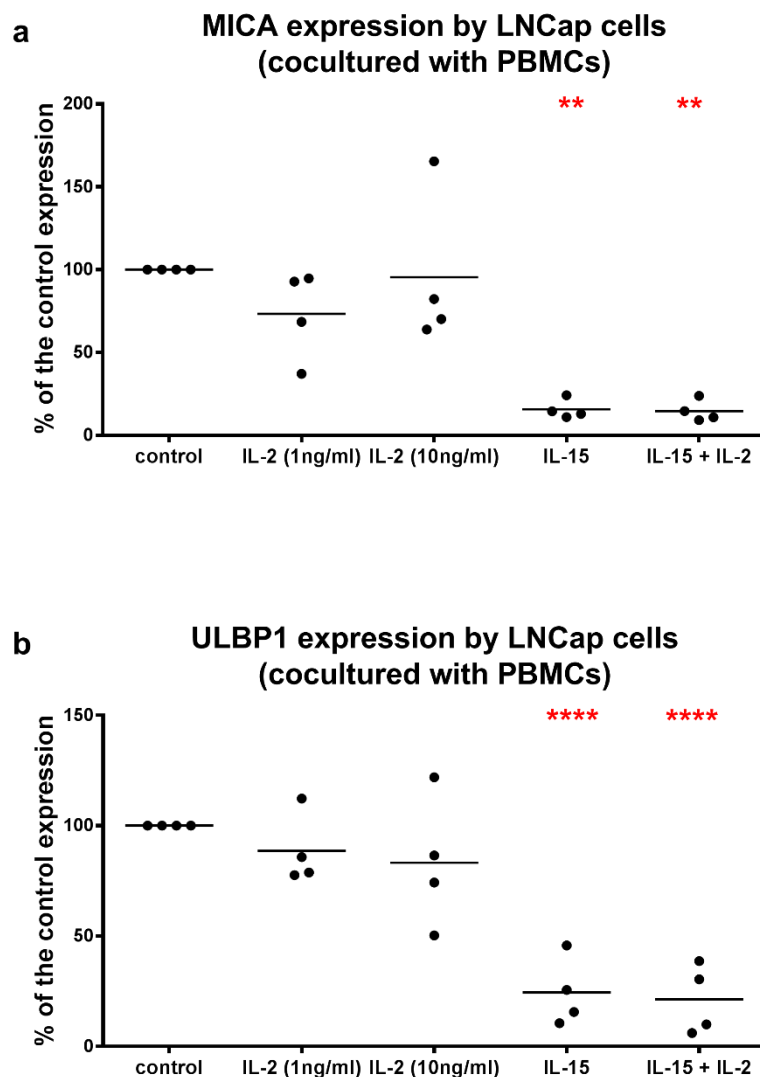


Figure 4.12 a,b: Expression of MICA and ULBP1 by LNCaP cells in cocultures with PBMCs. LNCaP cells were cocultured with non-adherent PBMCs cells in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) ( $n=4$ ,  $**p<0.01$ ,  $****p<0.0001$ , 1-way ANOVA).

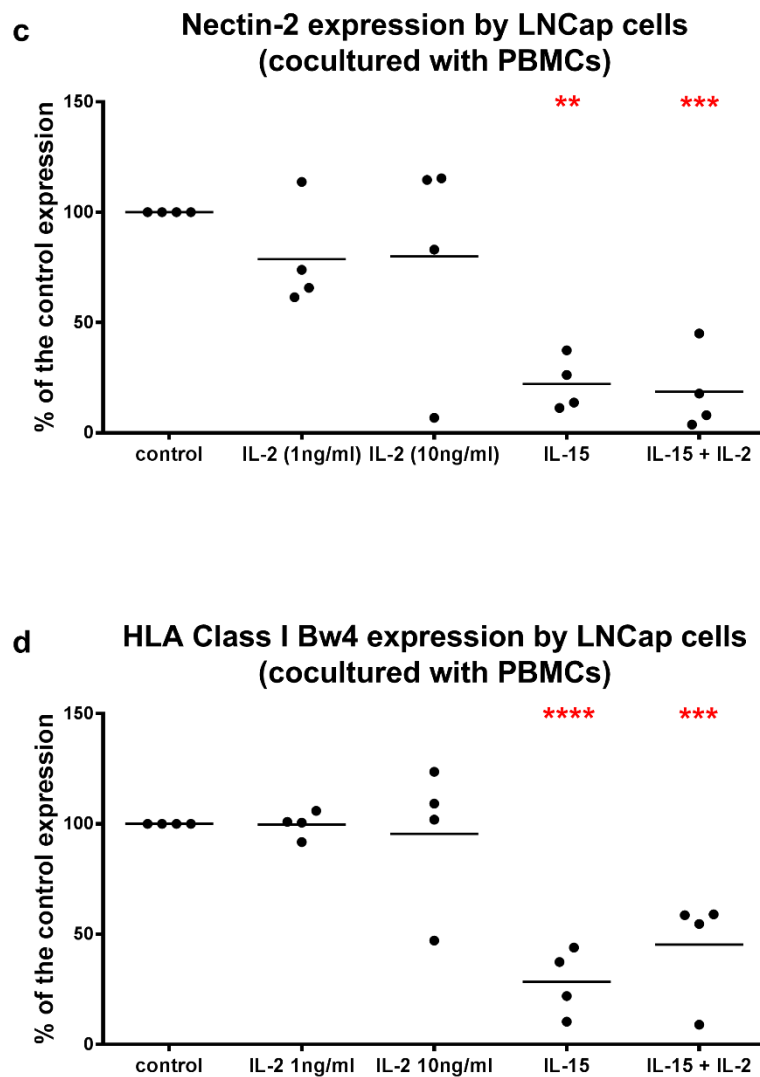


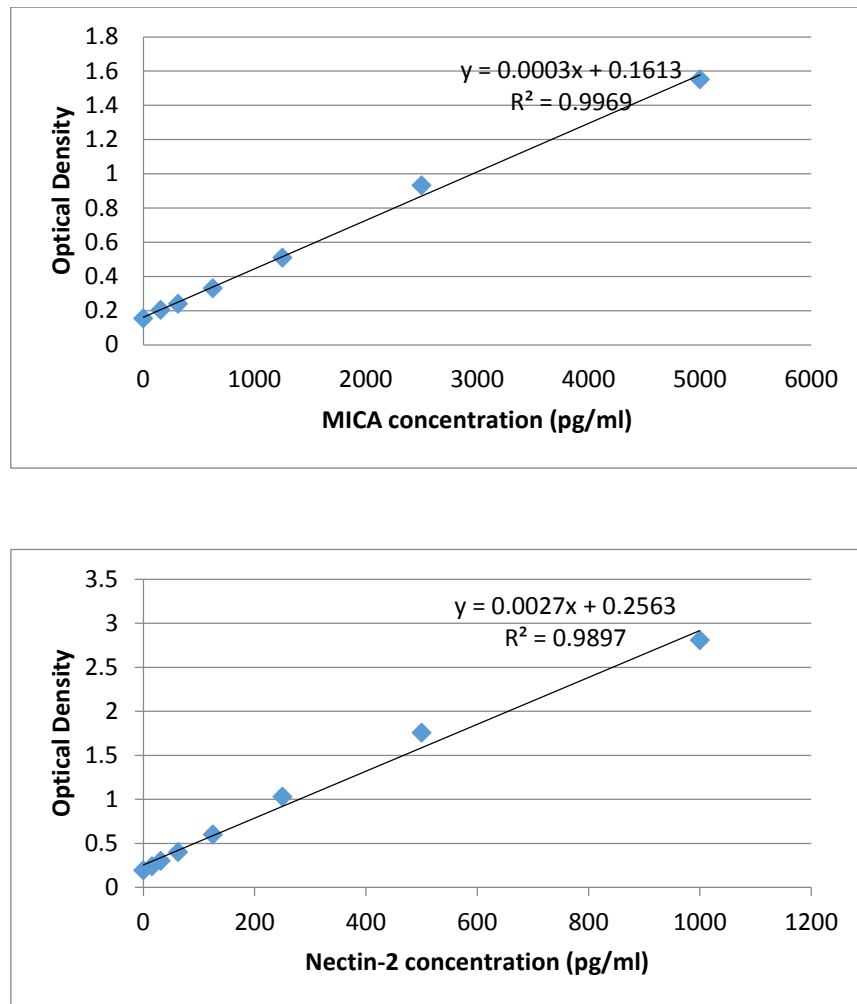
Figure 4.13 c,d: Expression of Nectin-2 and HLA Class I Bw4 by LNCaP cells in cocultures with PBMCs. LNCaP cells were cocultured with non-adherent PBMCs cells in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=4, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, 1-way ANOVA).

#### 4.2.4 Soluble expression of the Nectin-2 and MICA tumour cell ligands in the supernatants of the cocultured PCa/NK cells

In order to determine whether the downregulated expression of the above ligands (MICA, Nectin-2) in the presence of non-adherent PBMCs and IL-15 was due to increased ligand shedding, supernatants were collected from the allogeneic coculture model of non-adherent PBMCs with both PC-3 and LNCaP cell lines, in the presence or absence of the PBMCs, in the presence or absence of IL-15 and/or IL-2 cytokines. The supernatants were then tested by ELISA for the presence of MICA, ULBP1 and Nectin-2. Shedding of MICA and Nectin 2 was also investigated in cocultures of PC-3 cells with non-adherent PBMCs although surface expression of these ligands was not significantly altered.

There was no ULBP1 expressed in the supernatants of cocultures of PC-3 or LNCaP with non-adherent PBMCs, with or without cytokines; hence their results are not mentioned here.

The MICA and Nectin-2 ELISA standard curves are shown in figure 4.14 for the supernatants collected from the PC-3 and non-adherent PBMCs coculture model.



**Figure 4.14:** Standard curves of the MICA and Nectin-2 ELISAs. ELISAs were performed for the detection of MICA and Nectin-2 expression in the supernatants of cocultures of non-adherent PBMCs with PC-3 cells, in the absence or presence of IL-2 (1 or 10ng/ml) or IL-15 (2.5ng/ml) cytokines.

Interestingly, there was a significant decrease observed in the soluble expression of MICA in the presence of IL-2 in the cocultures of non-adherent PBMCs and PC-3 cells. However, the decrease was much greater in the presence of IL-15 ( $n=7$ ,  $**p<0.01$ ,  $****p<0.0001$ , 1-way ANOVA). Culturing PC-3 cells alone, with or without cytokines, did not affect expression of the soluble MICA (Figure 4.15).

No significant difference was observed in the Nectin-2 expression, neither in the cocultures with non-adherent PBMCs, nor in the PC-3 cells alone. The concentration of Nectin-2 in the supernatants of PC-3 cells when cultured alone, was lower than that in the cocultures with PBMCs, however the difference was not significant ( $n=4$ ,  $p>0.05$ , 1-way ANOVA) (Figure 4.15).

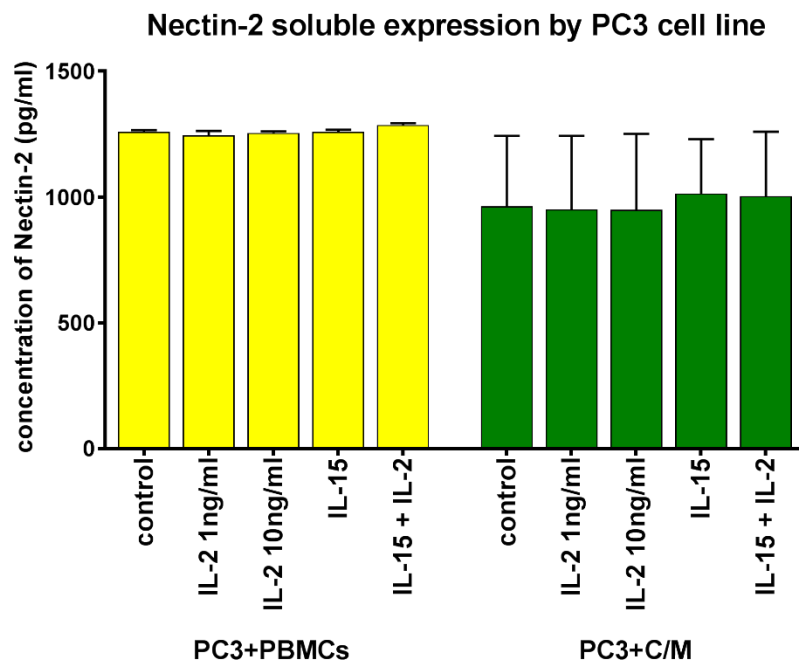
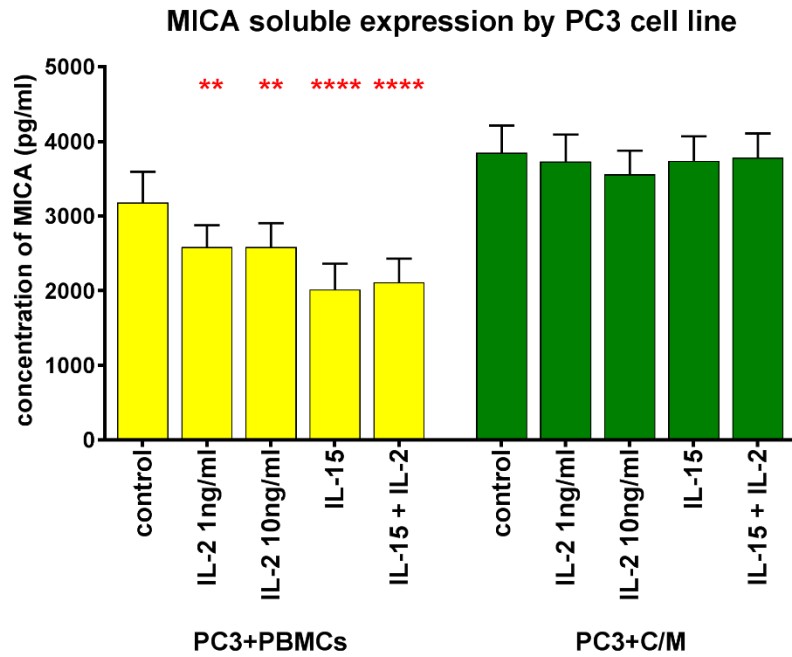
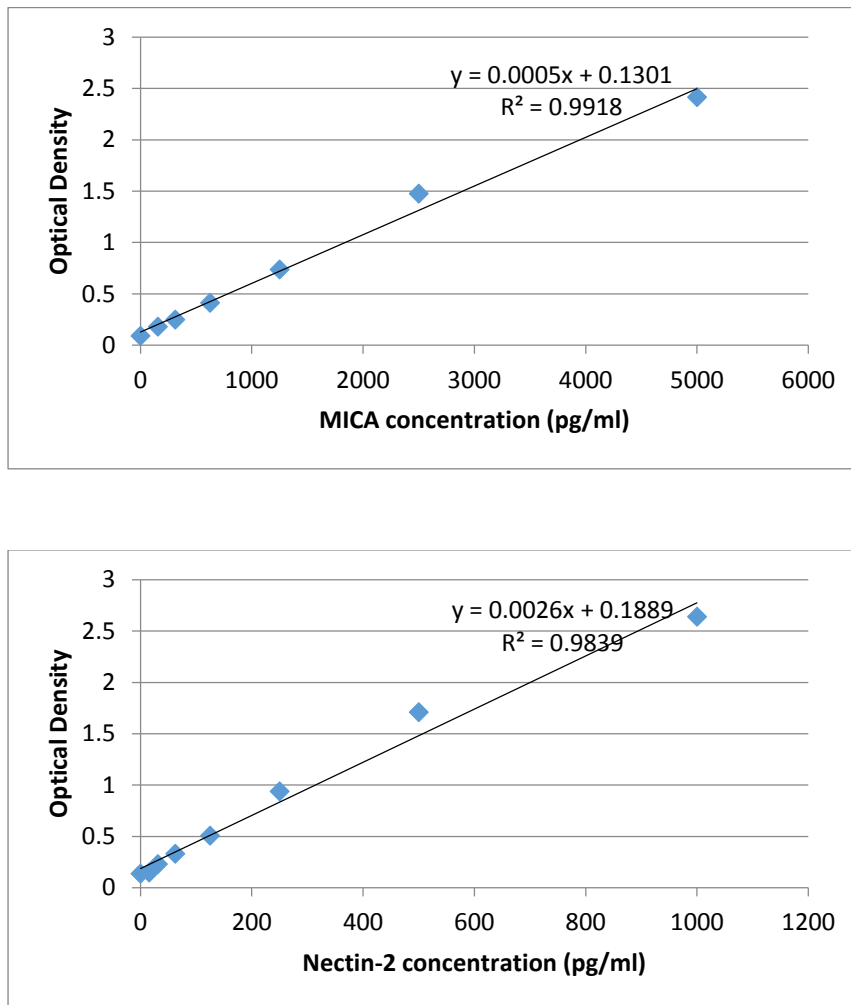


Figure 4.15: MICA and Nectin-2 soluble expression in supernatants of cocultures of PC-3 cells with PBMCs. ELISAs were performed for the detection of soluble MICA or Nectin-2 in the supernatants of the allogeneic cocultures, with or without non-adherent PBMCs and PC-3 cell lines, with IL-2 (1 or 10ng/ml) or IL-15 (2.5ng/ml) or without cytokines. MICA: n=7, \*\*p<0.01, \*\*\*\*p<0.0001, 1-way ANOVA. Nectin-2: n=4, p>0.05, 1-way ANOVA.

The MICA and Nectin-2 ELISA standard curves are shown in figure 4.16, for which the supernatants collected from the LNCaP and non-adherent PBMCs coculture model were tested.



**Figure 4.16: Standard curves of the MICA and Nectin-2 ELISAs.** ELISAs were performed for the detection of MICA and Nectin-2 expression in the supernatants of cocultures of non-adherent PBMCs with LNCaP cells, in the absence or presence of IL-2 (1 or 10ng/ml) or IL-15 (2.5ng/ml) cytokines.

There was a highly significant decrease observed in the soluble expression of MICA in the presence of IL-15 in the cocultures of non-adherent PBMCs with LNCaP cells, when compared to control and IL-2 (n=4, \*\*\*p<0.001, 1-way ANOVA); no effect on the MICA soluble expression was observed when LNCaP cells were cultured alone with or without cytokines (Figure 4.17).

No significant difference was observed in the Nectin-2 soluble expression, neither in the cocultures with non-adherent PBMCs, nor in the LNCaP cells alone (Figure 4.17). The concentration of Nectin-2 in the supernatants of LNCaP cells, when cultured alone, was lower than that in the cocultures with PBMCs, however the difference was not significant (n=4, p>0.05, 1-way ANOVA).



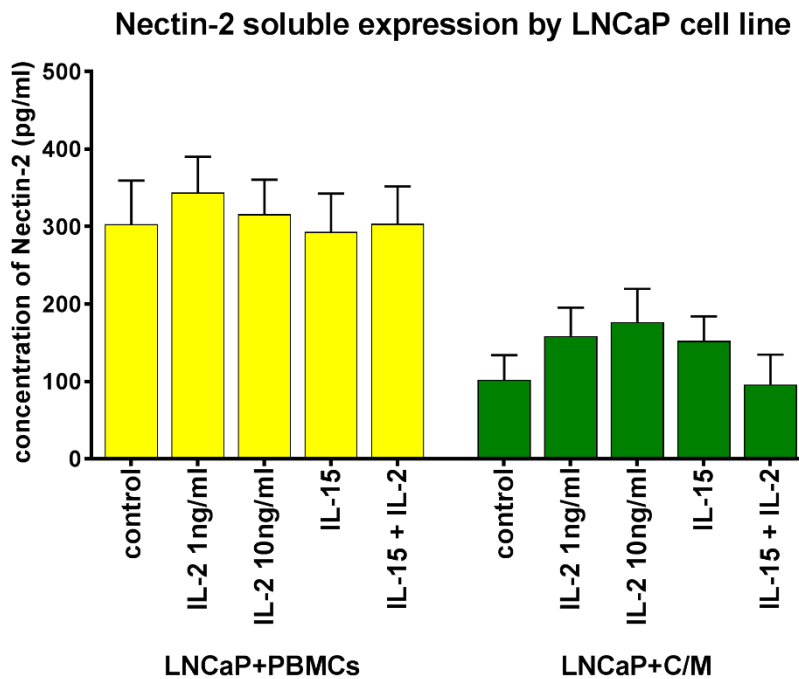
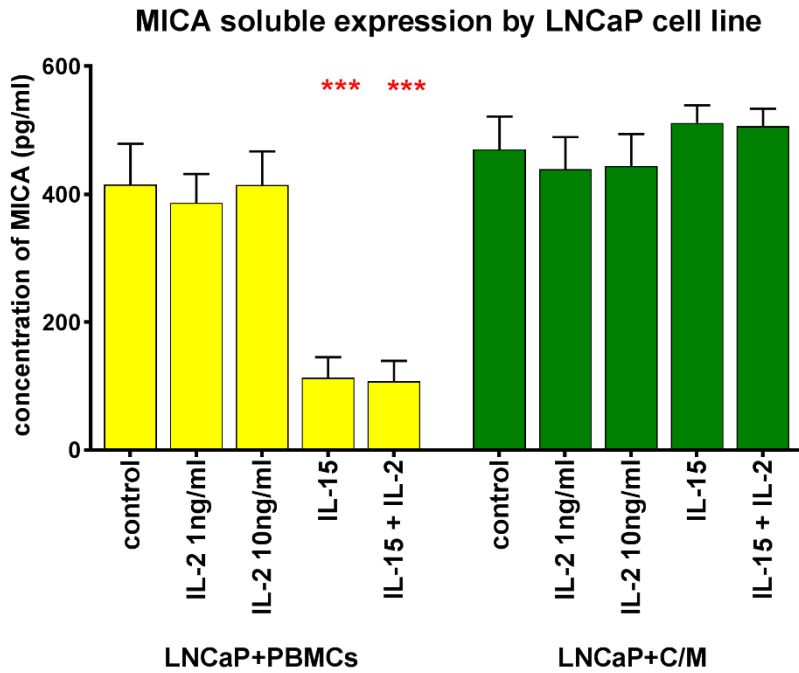


Figure 4.17: MICA and Nectin-2 soluble expression in supernatants of cocultures of LNCaP cells with PBMCs. ELISAs was performed for the detection of soluble MICA or Nectin-2 in the supernatants of the allogeneic cocultures, with or without non-adherent PBMCs and LNCaP cell lines, with IL-2 (1 or 10ng/ml) or IL-15 (2.5ng/ml) or without cytokines (n=4, p>0.05, 1-way ANOVA).

#### 4.2.5 Blocking of NKG2D and MICA in the *in vitro* allogeneic coculture model of non-adherent PBMCs and PCa cell lines

Due to the significant inhibitory effect of IL-15 on MICA shedding on PC-3 and LNCaP, NKG2D and its ligand, MICA, were blocked using neutralising antibodies in the *in vitro* allogeneic coculture model, in order to investigate whether NKG2D or MICA is most important in the NK activatory functions of IL-15.

Non-adherent PBMCs were cocultured with either PC-3 or LNCaP cell lines, in the absence or presence of IL-2 and IL-15 cytokines, with or without neutralizing antibodies against NKG2D and MICA or corresponding isotype controls. The NK and NKT cell expansions were measured as well as the NKG2D expression. MICA was not successfully blocked in our coculture system, hence the results are not shown. In addition, the PCa cell killing by NK and NKT cells was measured by Annexin/PI staining and the supernatants were also collected to check for the presence of shed MICA ligand.

##### 4.2.5.1 NK and NKT cell expansion in cocultures of immune effector cells with PCa cell lines, in the presence of an NKG2D blocking antibody

Non-adherent PBMCs were cultured with or without PC-3 cells, in the absence or presence of IL-2 and IL-15, with or without aNKG2D antibody or isotype control; figure 4.18 shows the expansion of NK and NKT cells and the NKG2D surface expression per NK and NKT cell respectively. IL-15 greatly expands the NK and NKT cells, as it has been shown previously, even when the NKG2D receptor is blocked. However, NK cells in cocultures of non-adherent PBMCs and PC-3 cells are expanded more by IL-15, than NK cells in non-adherent PBMCs alone treated with IL-15 (n=4, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, 1-way ANOVA).

The percentage of NKG2D receptor per NK cell was substantially increased in the presence of IL-15, when compared to control, when PBMCs are cultured alone (n=4, \*p<0.05, 1-way ANOVA). Blocking NKG2D seems to be decreasing the expansion of NK cells in cocultures of

non-adherent PBMCs with PC-3 cells; this may be partly due to an increase in NKG2D in the presence of IL-15 or due to a decrease in the shed MICA.

The expression of NKG2D in the presence of the blocking antibody was significantly lower, both in the presence or absence of PC-3 cells, signifying that the receptor was successfully blocked and that the blocking of the receptor does not affect the NK cell expansion.

NKT cells were also substantially increased in the presence of IL-15, both in the presence and absence of PC-3 cells ( $n=4$ ,  $*p<0.05$ ,  $**p<0.01$ ,  $***p<0.001$ ,  $****p<0.0001$ , 1-way ANOVA).

The NKG2D expression by NKT cells in cocultures with PC-3 cells was significantly higher in the presence of IL-15, compared to that in the presence of aNKG2D ( $n=4$ ,  $*p<0.05$ , 1-way ANOVA) (figure 4.19).

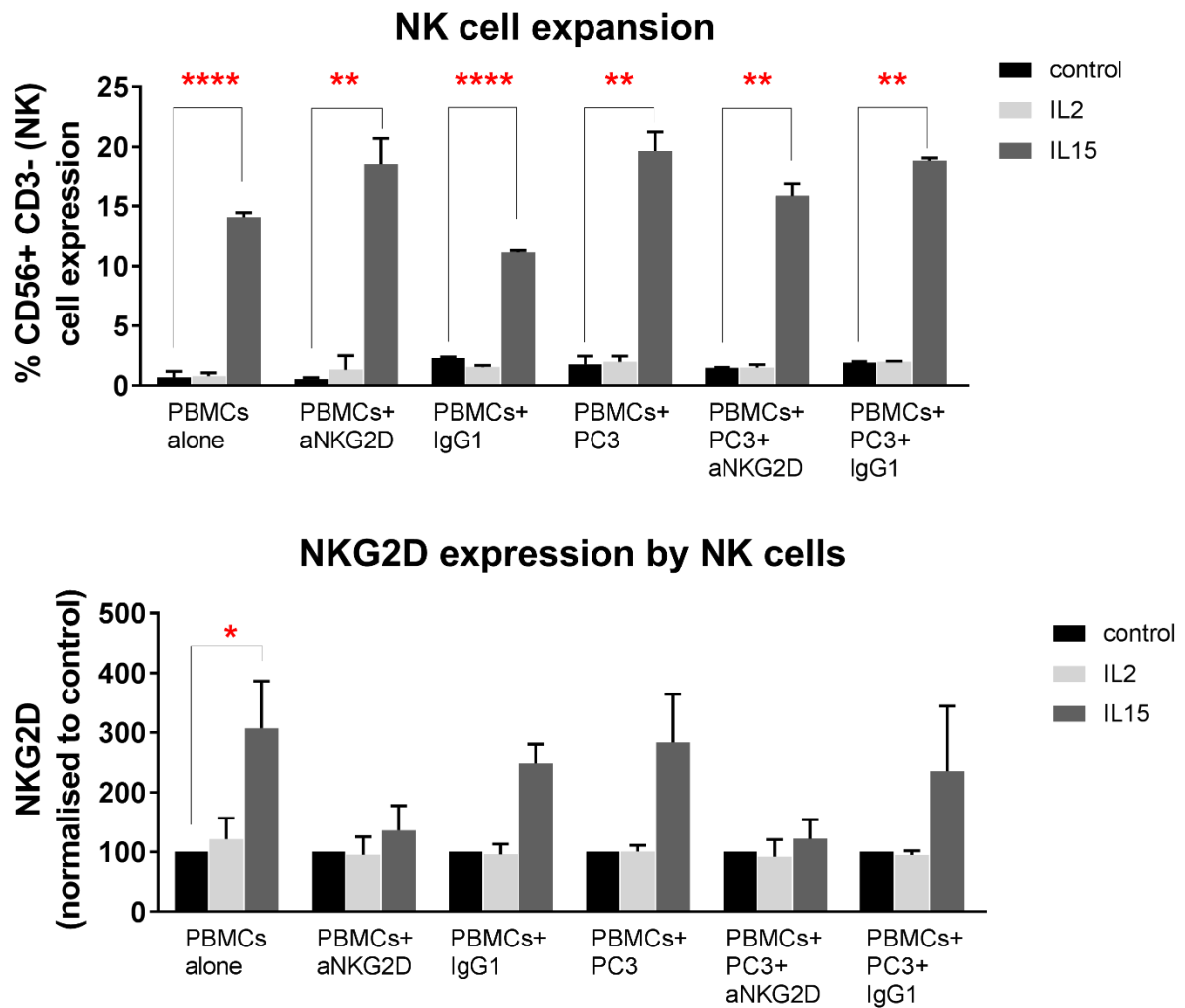


Figure 4.18: NKG2D blockade in cocultures of PBMCs with PC-3 cells: effects on NK cell expansion and NKG2D expression. NK cell expansion, as observed by CD56<sup>+</sup> CD3<sup>-</sup> cell expression and the NKG2D receptor expression per NK cell are shown, when non-adherent PBMCs were cultured alone, with or without PC-3 cells, with or without IL-2 (10ng/ml) and IL-15 (2.5ng/ml), with or without the addition of aNKG2D (10µg/ml) or its corresponding IgG1 isotype control (10µg/ml) (n=4, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, 1-way ANOVA).

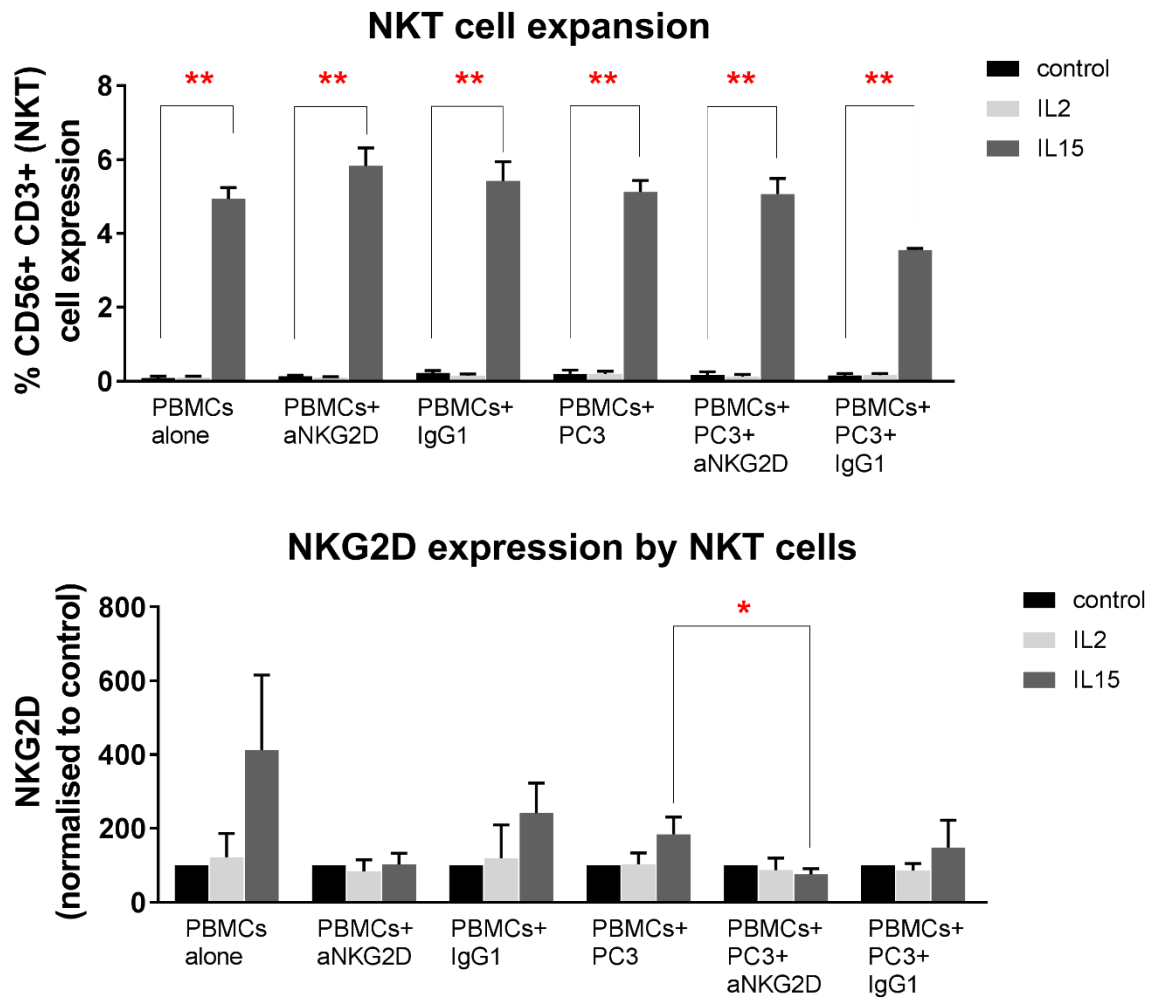


Figure 4.19: NKG2D blockade in cocultures of PBMCs with PC-3 cells: effects on NKT cell expansion and NKG2D expression. NKT cell expansion, as observed by CD56<sup>+</sup> CD3<sup>+</sup> cell expression and the NKG2D receptor expression per NKT cell are shown, when non-adherent PBMCs were cultured alone, with or without PC-3 cells, with or without IL-2 (10ng/ml) and IL-15 (2.5ng/ml), with or without the addition of aNKG2D (10µg/ml) or its corresponding IgG1 isotype control (10µg/ml) (n=4, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, 1-way ANOVA).

The same experiment was repeated in cocultures with LNCaP cells, as shown in figures 4.20 and 4.21. IL-15 highly expanded the NK and NKT cells, when non-adherent PBMCs were cultured on their own and this expansion was even higher in cocultures with LNCaP cells (n=4, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, 1-way ANOVA).

The presence of IL-15 increased the NKG2D expression by NK cells in cocultures of non-adherent PBMCs with LNCaP cells, whereas only when the PBMCs were cultured alone, IL-15 increased the expression of NKG2D by NKT cells (n=4, \*p<0.05, 1-way ANOVA).

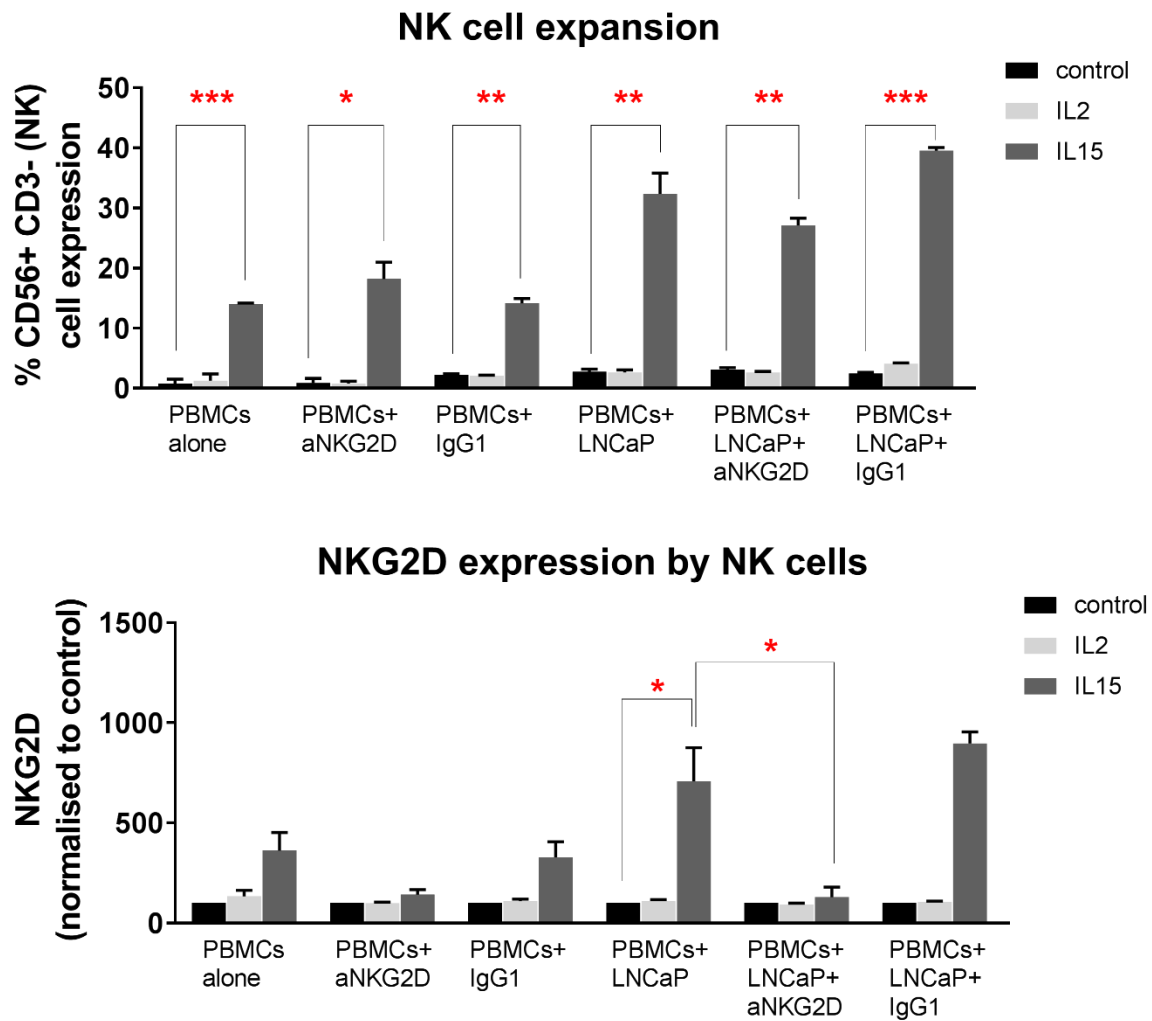


Figure 4.20: NKG2D blockade in cocultures of PBMCs with LNCaP cells: effects on NK cell expansion and NKG2D expression. The NK cell expansion, as observed by CD56<sup>+</sup> CD3<sup>-</sup> cell expression and the NKG2D receptor expression per NK cell were shown, when non-adherent PBMCs were cultured alone, with or without LNCaP cells, with or without IL-2 (10ng/ml) and IL-15 (2.5ng/ml), with or without the addition of aNKG2D (10μg/ml) or its corresponding IgG1 isotype control (10μg/ml) (n=4, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, 1-way ANOVA).

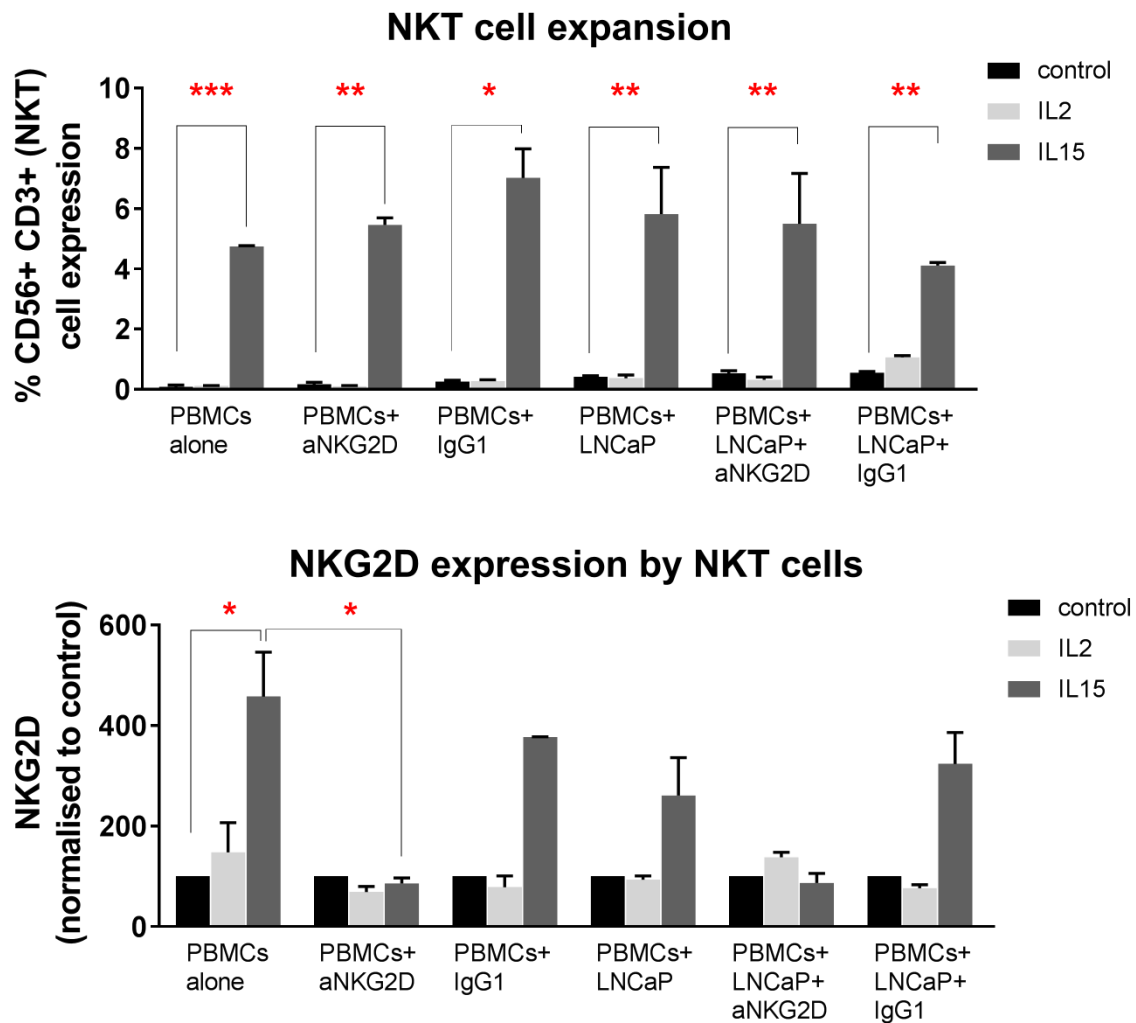


Figure 4.21: NKG2D blockade in cocultures of PBMCs with LNCaP cells: effects on NKT cell expansion and NKG2D expression. The NKT cell expansion, as observed by CD56<sup>+</sup> CD3<sup>+</sup> cell expression and the NKG2D receptor expression by these NKT cells is shown, when non-adherent PBMCs were cultured alone, with or without LNCaP cells, with or without IL-2 (10ng/ml) and IL-15 (2.5ng/ml), with or without the addition of aNKG2D (10μg/ml) or its corresponding IgG1 isotype control (10μg/ml) (n=4, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, 1-way ANOVA).



#### 4.2.5.2 PCa cell cytotoxicity by immune effector cells in cocultures of non-adherent PBMCs with PCa cell lines, in the presence of an NKG2D blocking antibody

The cytotoxic activity of non-adherent PBMCs towards the PCa cell lines (PC-3 and LNCaP) in the presence of an NKG2D blocking antibody was also assessed. In the cocultures of non-adherent PBMCs with LNCaP cells, with or without NKG2D blocking antibody and in the presence of IL-15, an increased amount of apoptotic cells was observed, by Annexin<sup>+</sup> cell staining, when compared to the control or IL-2 (Figure 4.22) (n=3, \*\*p<0.01, 1-way ANOVA). No significant difference was observed in the percentage of apoptotic cells with or without NKG2D blockade either in the presence or absence of cytokines. Similar results were observed in the cocultures with PC-3 cells, hence they are not shown here.

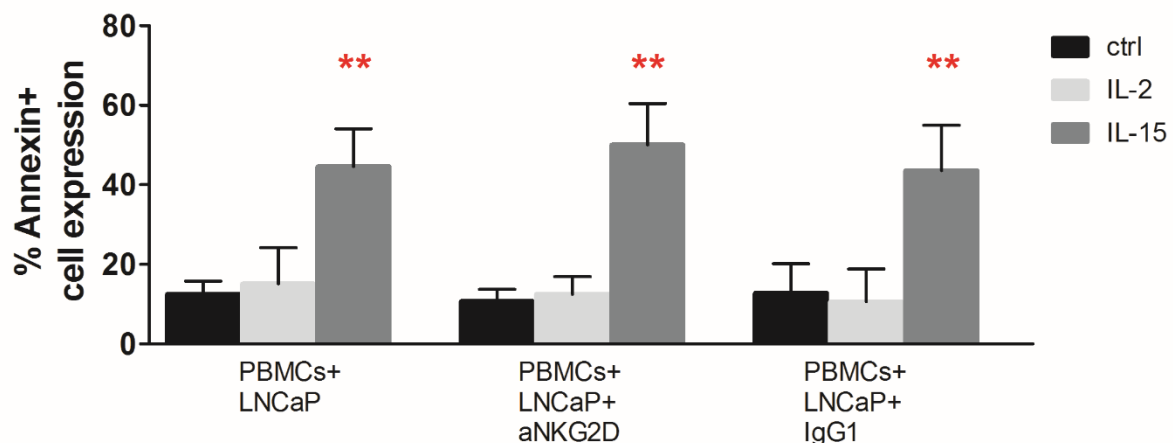
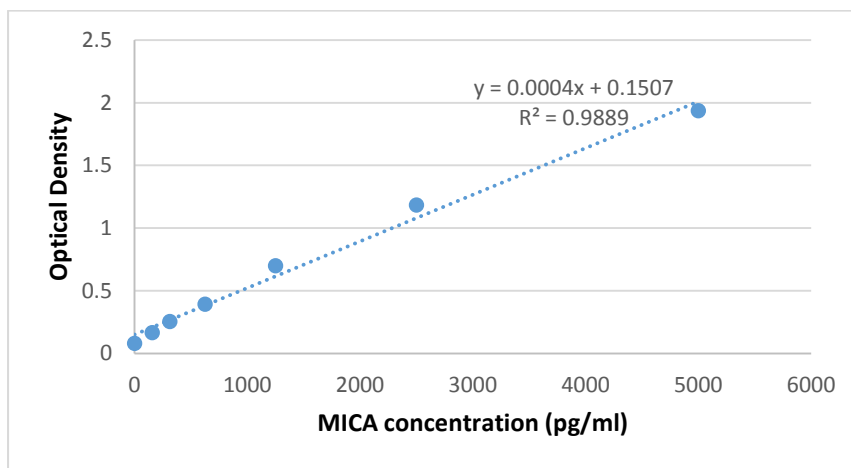


Figure 4.22: Annexin<sup>+</sup> cell expression by LNCaP cells in cocultures with PBMCs in the presence or absence of aNKG2D. Non-adherent PBMCs were cocultured with LNCaP cells, in the presence or absence of IL-2 (10ng/ml) and IL-15 (2.5ng/ml), in the presence or absence of aNKG2D (10μg/ml) or its corresponding IgG1 isotype control (10μg/ml) (n=3, \*\*p<0.01, 1-way ANOVA).

#### 4.2.5.3 Investigation of MICA soluble expression in the supernatants of cocultures of non-adherent PBMCs with PCa cell lines, in the presence of and NKG2D and MICA blocking antibodies

The supernatants from the cocultures of non-adherent PBMCs with PCa cell lines, with or without blocking antibody against NKG2D and MICA, were collected and tested by ELISA for the presence of soluble MICA. Even if MICA was not blocked in the phenotyping and cell killing experiments, the supernatants from the samples where MICA was blocked were still collected and tested on the ELISA.



**Figure 4.23: Standard curve of the MICA ELISA.** Soluble MICA in the supernatants of cocultures of non-adherent PBMCs with PCa cells, in the absence or presence of IL-2 (1 or 10ng/ml) or IL-15 (2.5ng/ml) cytokines, in the presence or absence of blocking antibodies against MICA and NKG2D, was investigated by ELISA.

In the cocultures of non-adherent PBMCs with PC-3 cells, the presence of IL-15 decreased the expression of soluble MICA (n=3, \*p<0.05, \*\*p<0.01, 1-way ANOVA), as also shown previously on figure 4.15; however since this was a new set of *in vitro* experiments, this had to be repeated. When NKG2D was blocked, the soluble MICA concentration increased, however that increase was not significant (n=3, p>0.05, 1-way ANOVA) (Figure 4.24).

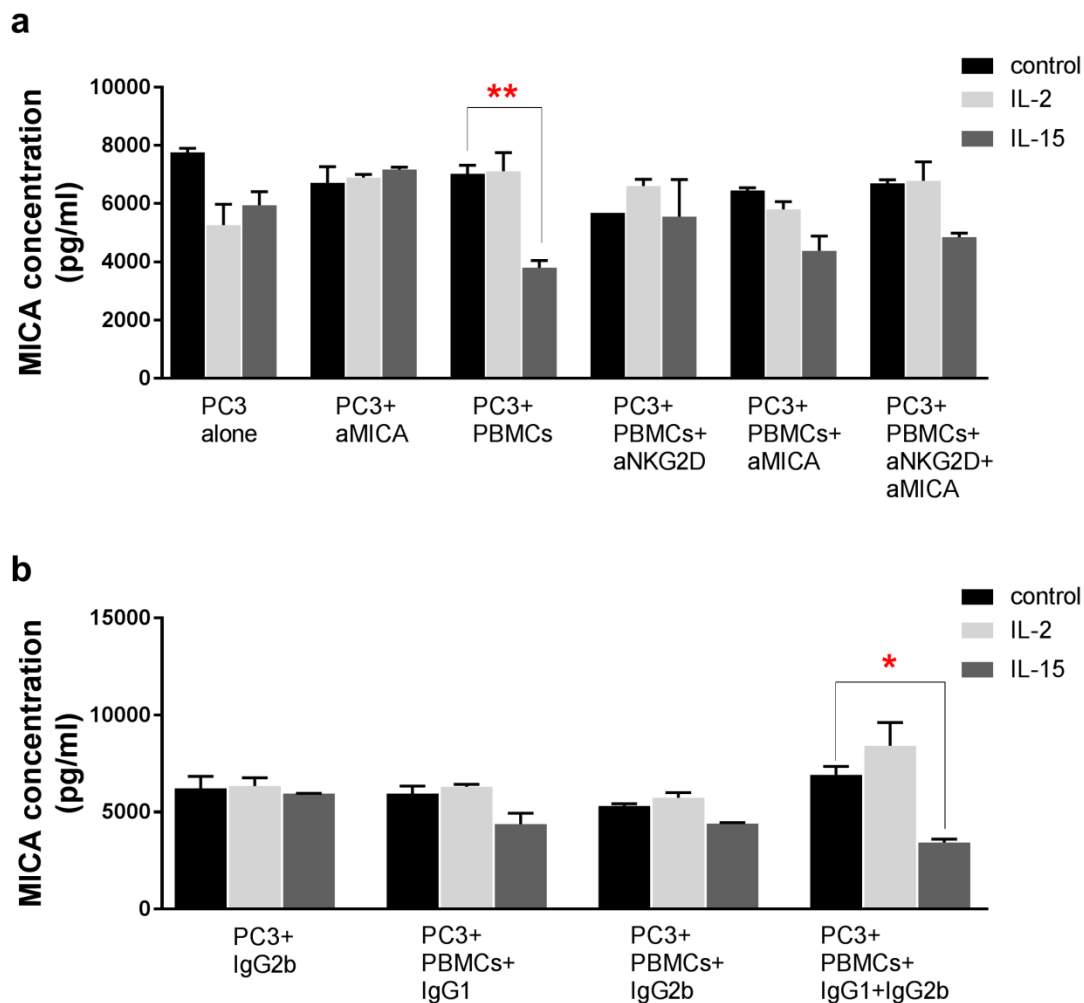


Figure 4.24: MICA soluble expression in supernatants of cocultures of PC-3 cells with PBMCs in the presence or absence of aNKG2D. ELISA was performed for the detection of soluble MICA in the supernatant of the allogeneic cocultures, of PC-3 cell line with or without non-adherent PBMCs, in the presence or absence of IL-2 (10ng/ml) or IL-15 (2.5ng/ml), **a**) with or without blocking antibodies against NKG2D (10µg/ml) and MICA (5µg/ml) or **b**) their corresponding IgG1 (10µg/ml) and IgG2b (5µg/ml) isotype controls respectively (n=3, \*p<0.05, \*\*p<0.01, 1-way ANOVA).

In cocultures of non-adherent PBMCs with the LNCaP cell line, IL-15, in the presence of non-adherent PBMCs, significantly downregulated the MICA soluble expression, when compared to control or IL-2. This decrease was consistent among all samples and was independent of the NKG2D or of the MICA blocking ( $n=3$ ,  $*p<0.05$ ,  $**p<0.01$ ,  $***p<0.001$ ,  $****p<0.00001$ , 1-way ANOVA) (Figure 4.25).

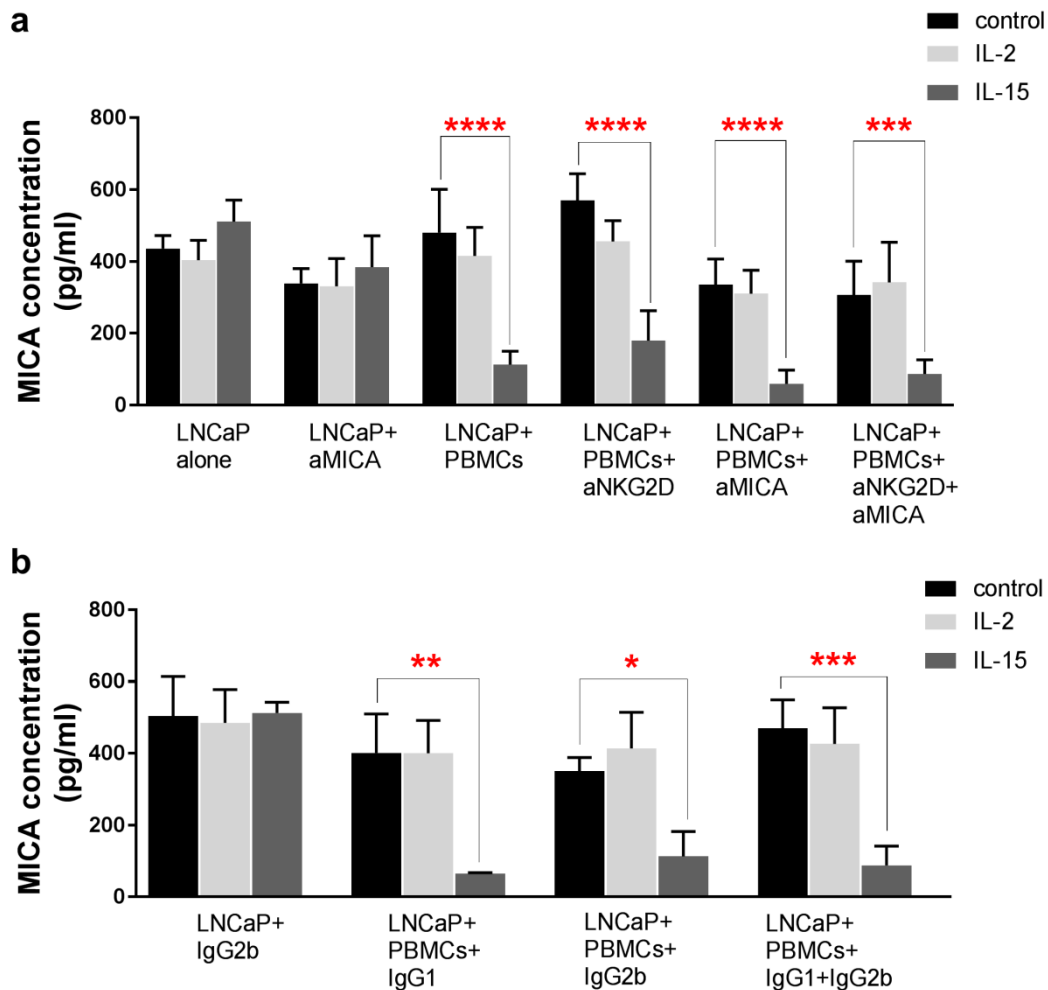


Figure 4.25: MICA soluble expression in supernatants of cocultures of LNCaP cells with PBMCs in the presence or absence of aNKG2D. ELISA was performed for the detection of soluble MICA in the supernatant of the allogeneic cocultures, of LNCaP cell line with or without non-adherent PBMCs, in the presence or absence of IL-2 (10ng/ml) or IL-15 (2.5ng/ml), **a**) with or without blocking antibodies against NKG2D (10 $\mu$ g/ml) and MICA (5 $\mu$ g/ml) or **b**) their corresponding IgG1 (10 $\mu$ g/ml) and IgG2b (5 $\mu$ g/ml) isotype controls respectively ( $n=3$ ,  $*p<0.05$ ,  $**p<0.01$ ,  $***p<0.001$ ,  $****p<0.00001$ , 1-way ANOVA).

### 4.3 Discussion

In order to assess the mechanism of action of IL-15 in our coculture model of immune effector cells with PCa cell lines, we focused on its ability to activate and expand NK cells; therefore the effects of IL-15 on NKR and their associated ligands on PCa cells were examined. Physiologically, low amounts of IL-2 should be present in the prostate; therefore we aimed to mimic the prostate microenvironment, by studying the effects of IL-15 in the presence of low concentration of IL-2, apart from IL-15 alone.

Initially the effects of IL-2 and IL-15 on NKR when non-adherent PBMCs were cultured alone were assessed; IL-2 did not affect expression of any of the receptors when compared to control (no cytokine), however in the presence of IL-15, the expression of KIR2DL2, NKp46 and DNAM-1 was highly upregulated, when compared to control or IL-2. These data are in compliance with existing literature, which suggests that IL-15 increases the expression of DNAM-1 (Hromadnikova, Li et al. 2016) as well as NKp30, NKp44 and NKp46 (de Rham, Ferrari-Lacraz et al. 2007).

Regarding the NKG2D receptor, one of the best-characterised and investigated aNKRs, different studies have found that IL-15 can either have no effect on its expression (Muntasell, Magri et al. 2010) or they can upregulate it, enhancing the cytotoxicity of NK cells (Sutherland, Chalupny et al. 2002, Wu, Higgins et al. 2004).

When non-adherent PBMCs were cocultured with PC-3 cells, the presence of IL-2 did not affect any of the receptors expressed, whereas in the presence of IL-15 NKG2D was upregulated, when compared to control and IL-2. In the cocultures with LNCaP cells the presence of IL-2 only, upregulated DNAM-1 expression by NK cells, whereas IL-15 downregulated NKp30 expression, but upregulated KIR2DL2 and DNAM-1 as well. Although a decrease in the expression of natural cytotoxicity receptors would be expected to result in a decreased activity of the NK cells, it is possible that the inhibition of NKp30 by IL-15 and not IL-2 is a more significant finding which may explain the effect of IL-15 on NK cell activity. However NK cells are activated to kill both PC-3 and LNCaP cells in the presence of IL-15 and therefore the significance of the decreased expression of NKp30 in cocultures with LNCaP and not PC-3 cells is not clear. Due to the complexity of the above controversial findings and

also due to contradicting literature on the effects of cytokines on these receptors, we decided to examine the corresponding ligands of these NKR on PCa cells.

None of the ligands' expression was significantly changed on the PCa cell lines when they were cultured without lymphocytes, in the presence of either IL-2 or IL-15 cytokines, when compared to control (see appendix 1). However, in cocultures of non-adherent PBMCs with PC-3 cells, the presence of IL-15 downregulated the expression of ULBP1 (ligand for the receptor NKG2D), when compared to control and IL-2. In cocultures with LNCaP cells, the presence of IL-15 (and not of IL-2) additionally highly downregulated the expression of MICA, Nectin-2 (for the receptors NKG2D and DNAM-1 respectively) and HLA Class-I Bw4 (for the receptor KIR3DL1).

The downregulation of MICA, Nectin-2 and ULBP1 on PCa cells cultured with lymphocytes in the presence of IL-15 is also a controversial finding. Ligands for NKG2D are thought to activate NK cells through NKG2D when expressed on tumour cells and downregulation of these ligands is thought to be associated with cancer progression (de Kruijf, Sajet et al. 2012). Furthermore, in two separate studies on coeliac disease patients, it was found that the overexpression of IL-15 in intestinal cells upregulated MICA, enhancing the cytotoxicity of the intraepithelial lymphocytes (IELs) in an NKG2D dependent manner (Mention, Ben Ahmed et al. 2003, Meresse, Chen et al. 2004). Another study showed that the dendritic cell (DC) upregulation of MICA in response to IL-15 contributes to a poor DC-NK cross-talk, resulting in dampened NK cell activation, during a Hepatitis C viral infection (Jinushi, Takehara et al. 2003).

In addition to the cell surface expression, some of these NKG2D ligands can be shed or excreted from cells, by protein cleavage or even in an exosome form; cancer cells are also thought to evade NK cell cytotoxicity by shedding of these ligands (Raulet, Gasser et al. 2013). Soluble MICA has been found in sera of patients with various cancer types, such as colorectal cancer (Jinushi, Hodi et al. 2006), liver cancer (Jinushi, Takehara et al. 2005), epithelial cancers (Groh, Rhinehart et al. 1999) and PCa (Wu, Higgins et al. 2004). Soluble forms of ULBP1, ULBP2 and ULBP3 were also found on the supernatants of gastric tumour cell lines, as well as in sera of patients with haematopoietic malignancies (Song, Kim et al. 2006,

Waldhauer and Steinle 2006). The presence of soluble ligands in sera of cancer patients may correlate with poor prognosis of the disease, as in the case of soluble ULBP2 which correlated with poor prognosis of melanoma (Jinushi, Hodi et al. 2006).

Decreased surface expression of the MICA, ULBP1, Nectin-2 and HLA-I Bw4 ligands in the presence of IL-15 in the coculture model were found, hence it was hypothesised that IL-15 might also be inhibiting secreted forms of these ligands which resulted in an increase in NK cell activity. It was therefore investigated whether the presence of IL-15 was modifying the shedding of these ligands, by collecting supernatants from the allogeneic coculture models of non-adherent PBMCs with PC-3 or LNCaP cells.

It was observed that IL-15 did not alter the soluble expression of Nectin-2 in the presence or absence of PBMCs compared to control or IL-2, whereas in supernatants from cocultures of lymphocytes with either PC-3 or LNCaP, IL-15 was inhibiting the secreted form of MICA, when compared to control or IL-2, unlike in cultures of PC-3 or LNCaP cells alone. LNCaP cells seem to expand NK cells better than PC-3 cells in the presence of IL-15. This may be because there is less MICA shed from LNCaP cells, and this is decreased by IL-15.

NKG2D and MICA were subsequently blocked in the *in vitro* allogeneic system in order to investigate whether that would affect the NK cell expansion and cytotoxicity towards the PCa cells. Supernatants were collected so that soluble expression of MICA was studied, when NKG2D and MICA were blocked.

Despite using several different concentrations of MICA blocking antibody that were suggested by the purchasing company and by literature, the MICA was not blocked in the allogeneic coculture model, which could be due to the poor antibody production; due to shortage of time, testing of more neutralising antibodies was not possible. However, NKG2D was successfully blocked when non-adherent PBMCs were cultured alone and in the presence of PC-3 or LNCaP cells. The presence of IL-15 significantly expanded both NK and NKT cells when compared to control or IL-2, in the absence or presence of PCa cells. As it was also shown in previous experiments, the presence of IL-15 and of the PCa cell lines increases the expansion of NK and NKT cells even more, when compared to cultures of immune effector cells alone.

We were unable to replicate our initial experiments (figure 4.7) where NKG2D expression was significantly increased in the presence of IL-15, when non-adherent PBMCs were cocultured with PC-3 cells, with that shown in figure 24a; despite observing an increase in the expression, significance is not reached, but with a few more experimental repeats, the significance could be reached.

Additionally, on figure 4.10h the NKG2D expression by NK cells in the presence of IL-15 in cocultures with LNCaP cells seems to be similar to those of the control, however in the blocking experiments (figure 4.18), IL-15 seems to upregulate the NKG2D expression. From these results with the two cell lines, there is a trend observed, where IL-15 increases the NKG2D expression by NK cells, but in some cases the results have not reached significance, which indicates that more experiments should be done, as donor to donor, the amount of NK cells and of NKRs varies.

Blockade of NKG2D seemed to have been successful, since the expression of NKG2D by NK or NKT cells was minimal, both when PBMCs were cultured alone and in cocultures with PCa cell lines and the neutralising aNKG2D antibody. The addition of aNKG2D neutralising antibody did not affect the expansion of NK or NKT cells in cocultures of non-adherent PBMCs with PC-3 or LNCaP cells in the presence of IL-2 or IL-15. It is important to note however, that when aNKG2D antibody was added in the cocultures of non-adherent PBMCs with either of the cell lines, there was a decrease of NK expansion observed, compared to cocultures and IL-15 where NKG2D was not blocked.

The blockade of NKG2D did not affect the NK cytotoxicity towards the PCa cells; IL-15 increased the percentage of apoptotic cancer cells in cocultures with PC-3 or LNCaP cells, with or without the NKG2D blocking antibody, when compared to control or IL-2. Therefore the IL-15 mediated increase in NK cell cytotoxicity towards the tumour cells is not affected by NKG2D.

As mentioned, MICA was not successfully blocked on the PCa cell surface in cultures of PCa cells alone or in the cocultures with non-adherent PBMCs, however the supernatants from these samples were collected and also examined for the soluble expression of MICA.

When PC-3 cells were cultured alone, there was a non-significant decrease observed in the MICA soluble expression in the presence of IL-2 and IL-15 compared to control. However, in



the presence of non-adherent PBMCs, the decrease in MICA concentration was substantially inhibited in the presence of IL-15. Neither the blockade of NKG2D, nor that of MICA had any effect on the MICA soluble expression in the presence or absence of cytokines.

When LNCaP cells were cultured alone, there was a non-significant increase observed in the MICA soluble concentration in the presence of IL-2 and IL-15 compared to control. In the presence of PBMCs though, the MICA concentration was highly decreased in the presence of IL-15, when compared to control and IL-2, moreover that decrease was observed even in the presence of NKG2D and MICA blocking antibodies.

Taking all these results into account, it is proposed that the presence of IL-15 highly expands the NK cells and enhances the cytotoxicity towards the PCa cells in the allogeneic coculture model of non-adherent PBMCs with PCa cell lines, by upregulating the NKG2D receptor expression, with a concomitant decrease in the surface and soluble expression of its ligand MICA. An increased interaction between NKG2D and MICA may be occurring due to the decrease in shed MICA which acts to inhibit NK cell activation. Further experiments to block NKG2D or MICA through SiRNA or gene knockouts may be needed to further validate the role of NKG2D and MICA on this model. Also it may be worth exploring the role of the other ligands (e.g. HLA Class I Bw4), on the expansion and cytotoxic potential of IL-15 activated NK cells.

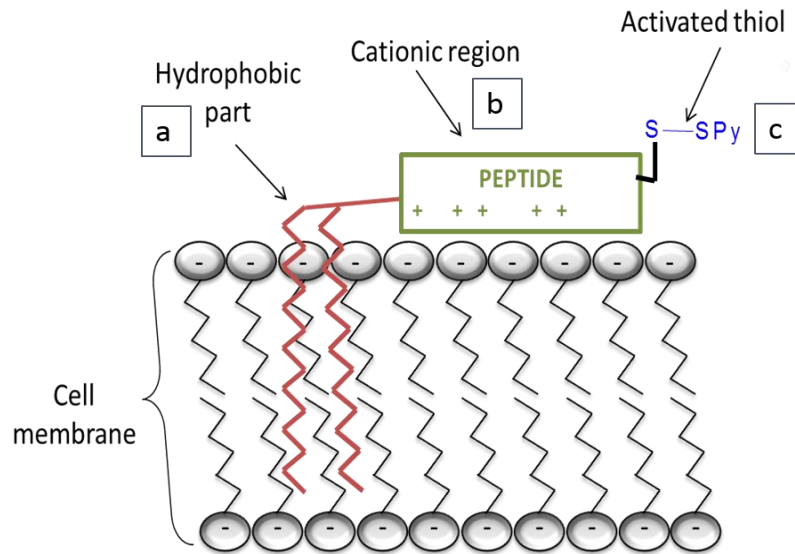
## CHAPTER 5

Cytotopic modification of recombinant human IL-15 and two antibodies against checkpoint inhibitor proteins and testing their activity *in vitro* and *in vivo*

## 5.1 Introduction

Non-cell based immunotherapies that are currently in use for many types of cancers (e.g. IL-2 for metastatic breast cancer) are administered systemically and can be extremely toxic and because of that they are only suitable for patients that are refractory to any other type of treatment (Fehniger, Cooper *et al.* 2002). For solid tumours, their localisation to the primary cancerous lesion however, would both increase their efficacy and lower their toxicity. Furthermore, when considering such agents for immunotherapies, it is vital to consider their action at the site of the lesion where the immune system is most compromised. Many cytokines will have no effect when injected into this immunosuppressive environment based on our preliminary *in vitro* coculture data.

Dr Richard Smith at King's College London has developed a unique technology that can localise any protein or peptide to cell membranes. This technique is known as cytotopic modification and can be used to construct proteins or peptides with amphiphilic (peptides containing both hydrophobic and hydrophilic regions) peptide-tails for tumour localisation (Smith and Smith 2001). Briefly, the protein of interest is attached to a "tail" molecule consisting of a myristoyl group and a cationic peptide sequence terminated with an activated thiol group that can link to free cysteines in the protein of interest (Smith and Smith 2001) (Figure 5.1). The technology has been successfully used to create a tailed CR1-fragment (APT070, Mirococept), which tethers to cell membranes and inhibits complement *in situ* more effectively than soluble CR1 (Patel, Smith *et al.* 2006); Mirococept is now in phase II kidney transplant trials.



**Figure 5.1: Illustration of the tail.** The ‘tail’ is comprised of (a) two myristoyl chains, that can build-in spontaneously to the hydrophobic portion of the cell membrane, which are attached to (b) a positively charged peptide: that interacts with the negatively charged hydrophilic portion of the cell membrane, securing the tail binding to the membrane and (c) a thiol group, which connects the tail to the peptide of interest (IL-15).

This cytotopic technology can be applied to immunotherapeutic proteins such as cytokines so that these can be localised within tumour lesions and break tolerance within this immunosuppressive microenvironment. Such an approach can have two advantages – firstly – systemic toxicity of these agents is avoided. Secondly, less of these agents are required to induce a potent immune response. This has been previously seen when IL-2 was injected *in situ* into melanoma skin lesions in patients resulting in the clearance of distant metastases (Johnson, Lum *et al.* 2008).

Based on the above, the aim of this project was to determine the efficacy of novel immunotherapeutics both as monotherapy and in combination, on the ability of NK and NKT cells to kill prostate tumour cells.

Three agents: IL-15 and two immune checkpoint inhibitor antibodies named PTL033 and PTL035 (according to the nomenclature on our laboratory), were chosen to be studied in the context of immunotherapy for PCa. They were chosen due to previous studies showing their effectiveness in treating PCa in preclinical studies using mice (Yu, Steel *et al.* 2012), and in the

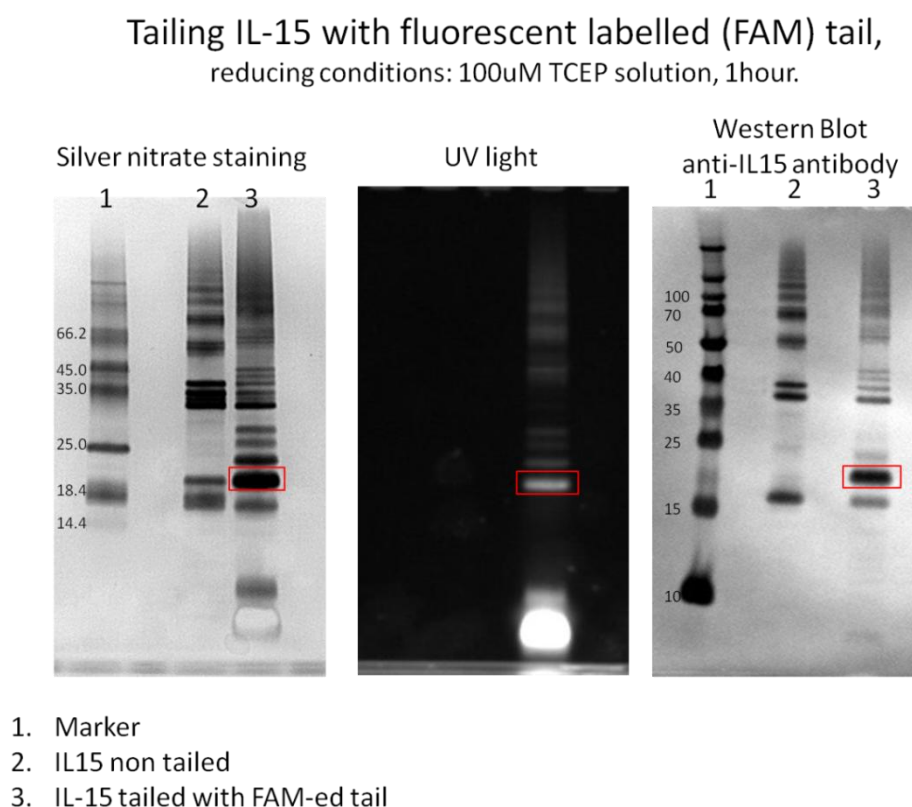
clinic. In our preliminary data we have also shown that IL-15 is the only cytokine from a panel of therapeutic Th1 cytokines that can induce the expansion of NK, NKT and CD8 T cells in the presence of PCa cells.

Dr Dorota Smolarek from the Protein Therapeutics Laboratory created a novel form of IL-15, so-called tailed IL-15, and the two immune checkpoint inhibitor antibodies using Dr Richard Smith's unique technology.

## 5.2 Results

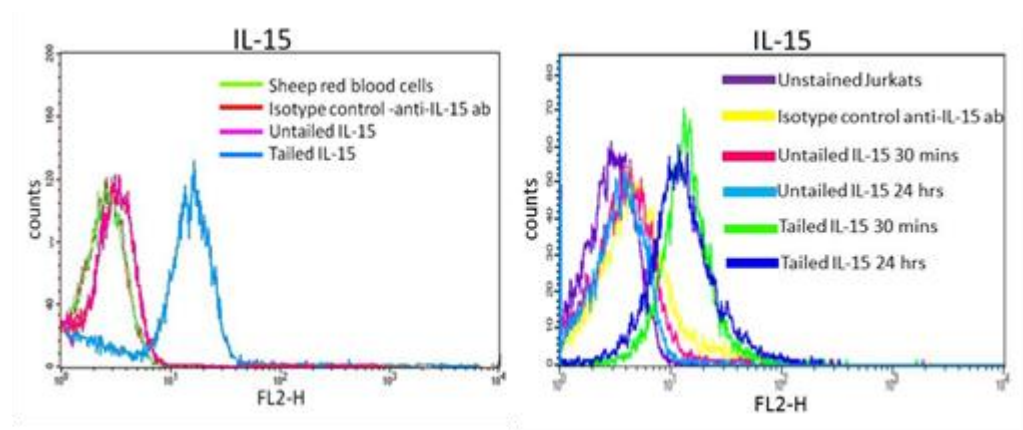
### 5.2.1 Confirmation of the cytototopic modification and of the cell membrane tethering of a) IL-15 and of b) the immune checkpoint inhibitor antibodies

a) The human IL-15 tailing was confirmed by a variety of methods: by observing a molecular mass shift in gel electrophoresis of the untailed and tailed protein, using a tail that had been labelled with a fluorophore - FAM (Carboxyfluorescein) and by western blot analysis using an antibody to IL-15 that recognises properly folded protein (Figure 5.2).



**Figure 5.2: Visualisation of tailed IL-15:** Visualisation of tailed IL-15. Gel electrophoresis followed by silver nitrate staining, Western blot analysis and UV light were employed for the visualisation of the fluorescently labelled tail. The band circled in red represents the main tailed protein moiety.

The ability of tailed IL-15 to bind to cell membranes was shown using flow cytometry and sheep red blood erythrocytes or Jurkat cells. These cell types were chosen as they do not have receptors or proteins that can be bound by the untailed IL-15. Binding was assessed by flow cytometric analysis of the binding of IL-15 to these cells as detected by fluorophore labelled antibody to IL-15 (PE labelled anti-IL-15) (Figure 5.3).



**Figure 5.3: Confirmation of cell membrane binding of tailed IL-15** by flow cytometric analysis with staining detected by PE-labelled anti-IL-15. Little or no binding is seen with untailed IL-15 either on sheep red blood cells (Left panel) or Jurkats (right panel). Similar results were obtained with 30 mins or 24 hr incubation of tailed IL-15 on Jurkat cells (Right panel) showing that it can be retained on cell membranes through the tail portion of the molecule for a significant period of time. Internalisation is therefore slow allowing significant cell-surface binding and presentation for activity.

**b)** Murine antibodies against immune checkpoint inhibitor proteins were conjugated to the tail (prepared by formation of disulphide- bridge between the tail and murine antibodies using 2-iminothiolane, as described in section 2.2.4.2 and 2.2.4.3). In order to confirm that the cytotopic modification was achieved, the tailed protein was visualised using SDS-PAGE electrophoresis followed by staining with Safestain™ (GE Healthcare) or UV light, where tail was FAM labelled (figure 5.4).

To test the ability of the tailed PTL033 and PTL035 to bind to cell membranes Jurkat cells were used, a T-cell line which does not have on their surface any receptors or proteins that can be bound by these proteins. Binding was assessed by flow cytometric analysis of the binding of the two modified antibodies to Jurkat cells, as detected by fluorophore labelled antibodies to these proteins (Figure 5.5).

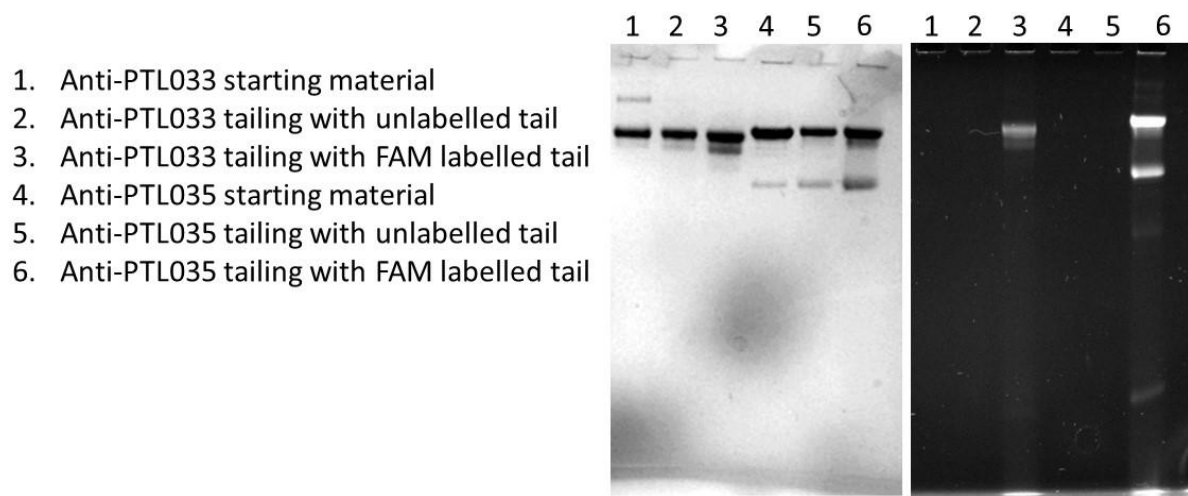


Figure 5.4: Visualisation of tailed PTL033 and PTL035 antibodies by SAFESTAIN (left) or UV light (right).

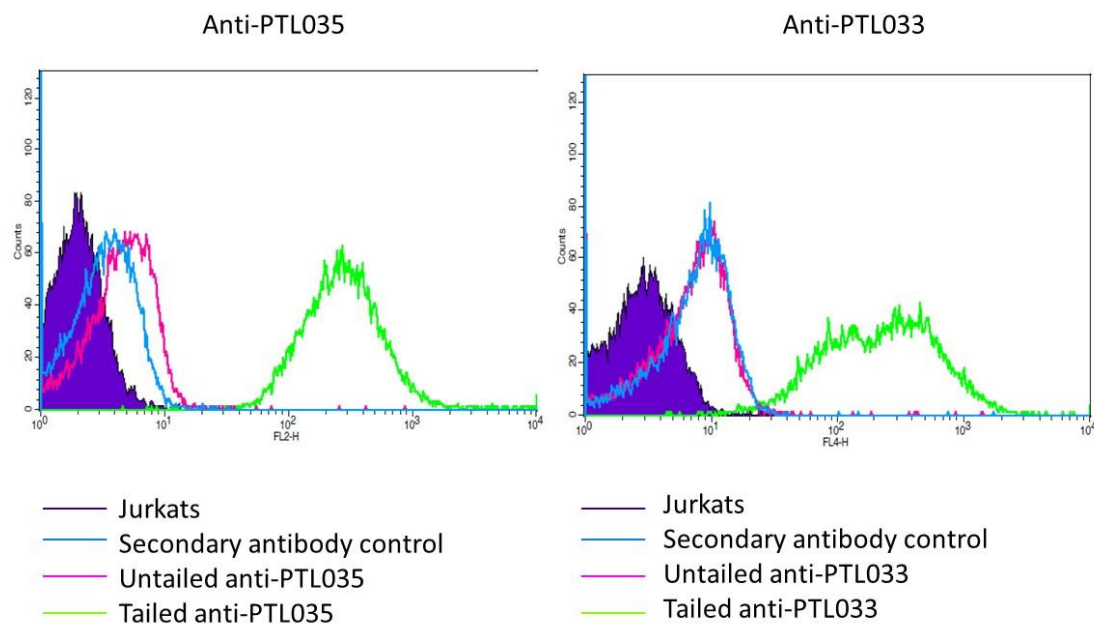


Figure 5.5: Confirmation of cell membrane binding of tailed PTL035 and PTL033. Flow cytometric analysis with staining detected by PE-labelled anti-rat IgG2b (in regards to PTL035) and APC labelled anti-hamster IgG (in regards to PTL033) showed little or no binding with untailed proteins, but significant binding with the tailed proteins.



### 5.2.2 Confirmation of activity of tailed IL-15, tailed PTL033 and tailed PTL035 *in vitro*

In order to measure the activity of tailed IL-15, a cell proliferation assay with a murine cytotoxic T cell line (CTLL-2) was performed. A higher CTLL-2 proliferation was observed and at lower concentrations of cytokine when incubating them with tailed IL-15, compared to the non-tailed (Figure 5.6).

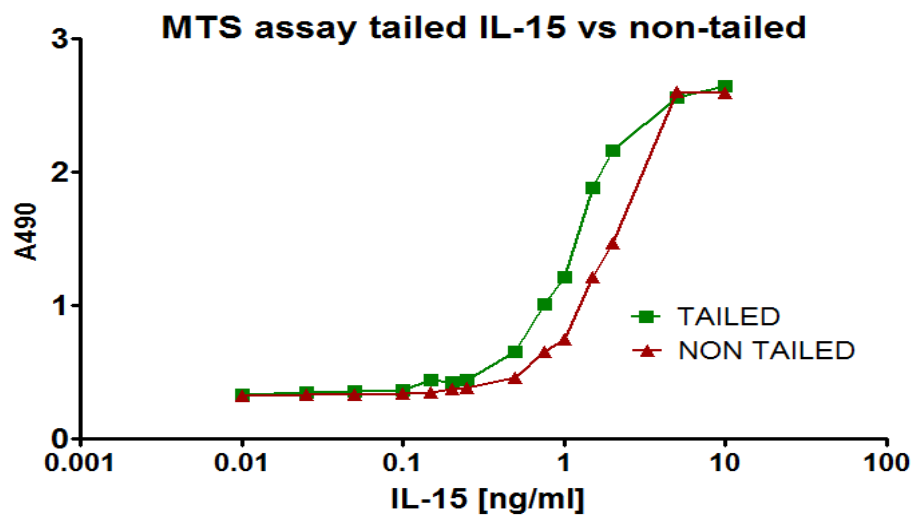
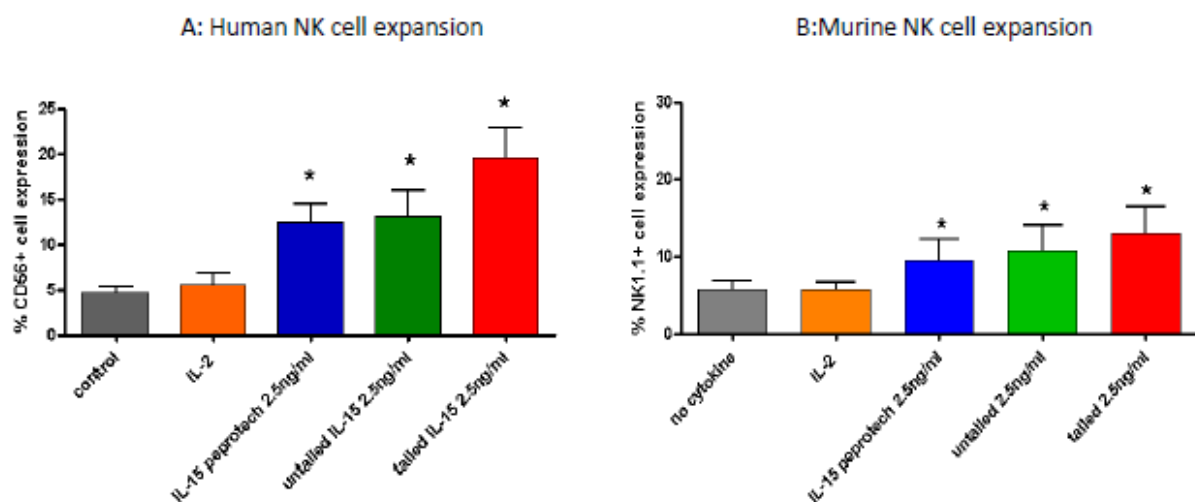


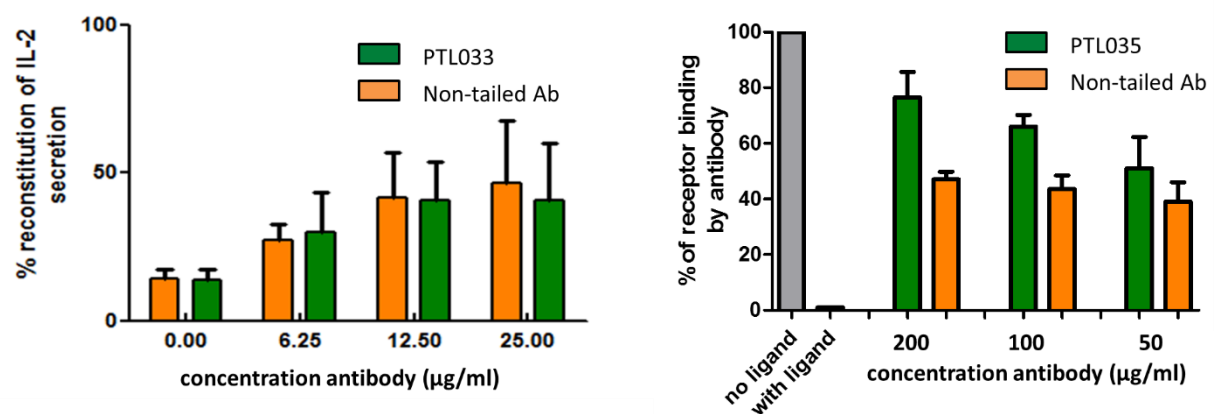
Figure 5.6: Confirmation of activity of tailed IL-15 by MTS. A cell proliferation assay (MTS) was performed in order to confirm the ability of tailed IL-15 to expand the CTLL-2 cell line. Higher proliferation is observed in the presence of tailed IL-15 protein.

The activity of tailed IL-15 was then confirmed using human and murine NK lymphocytes, which were incubated with tailed and non-tailed protein to induce their expansion. After 7 days of culture, the NK cell population was analysed by flow cytometry and it was shown that the tailed IL-15 has equivalent or greater ability to expand human NK cells (detected by staining of CD56<sup>+</sup>CD3<sup>-</sup> cells ) and murine NK cells (detected by staining of NK1.1<sup>+</sup>CD3<sup>-</sup> cells) compared with non-tailed IL-15 (Figure 5.7).



**Figure 5.7: NK cell expansion by tailed IL-15 confirming its activity.** Comparison of NK expansion in a human PBMC population (A) and a murine splenocyte population (B) treated for 7 days with IL-2 (100 units per ml) , Peprotech IL-15, untailed G-IL-15 and tailed G-IL-15 (2.5 ng/ml each). Tailed IL-15 has equivalent or better activity in expanding NK cells compared with untailed IL-15 from Genscript or a commercial preparation (Peprotech) (n= 3, \*p<0.05, 1-way ANOVA).

The activity of the tailed antibodies PTL033 and PTL035 was studied by measuring reconstitution of IL-2 secretion in the Jurkat cell based model (PTL033), or by prevention of binding between receptor and ligand on EL-4 cells (PTL035), using flow cytometry and recombinant proteins. It was observed that the tailed PTL033 has equivalent activity to the non-tailed antibody and the tailed PTL035 has a greater activity than non-tailed PTL035 (figure 5.8).



**Figure 5.8: Confirmation of activity of tailed PTL033 and of tailed PTL035.** Comparison of activity of tailed and untailed PTL033 antibody in restoring IL-2 secretion by activated Jurkat cells and of tailed and untailed PTL035 inhibition of blocking receptor and ligand interaction on EL-4 cells.

### 5.2.3 Confirmation of activity of tailed IL-15, tailed PTL033 and tailed PTL035 *in vivo* in C57BL/6 mice challenged with the TRAMP-C2 carcinoma cell line

The efficacy of tailed IL-15 to inhibit tumour growth was determined by an *in vivo* subcutaneous prostate cancer tumour challenge model in C57BL/6 mice. A previous study shows that IL-15 together with two antibodies to immune checkpoints can inhibit subcutaneous tumour growth in this model by up to 70% in 45 days (Yu, Steel et al. 2012). Initially, the optimal dose of injected TRAMP-C2 cells was determined (figure 5.9); three doses of different cell number concentrations were assessed for the quickest tumour growth. The concentration of  $1 \times 10^7$  cells per injection resulted in a quicker tumour formation; however it was not significantly different to the other doses. The dose of  $5 \times 10^6$  cells in total was used for further tumour cell challenges in mice as it has been previously used in literature (Kwon, Foster et al. 1999).

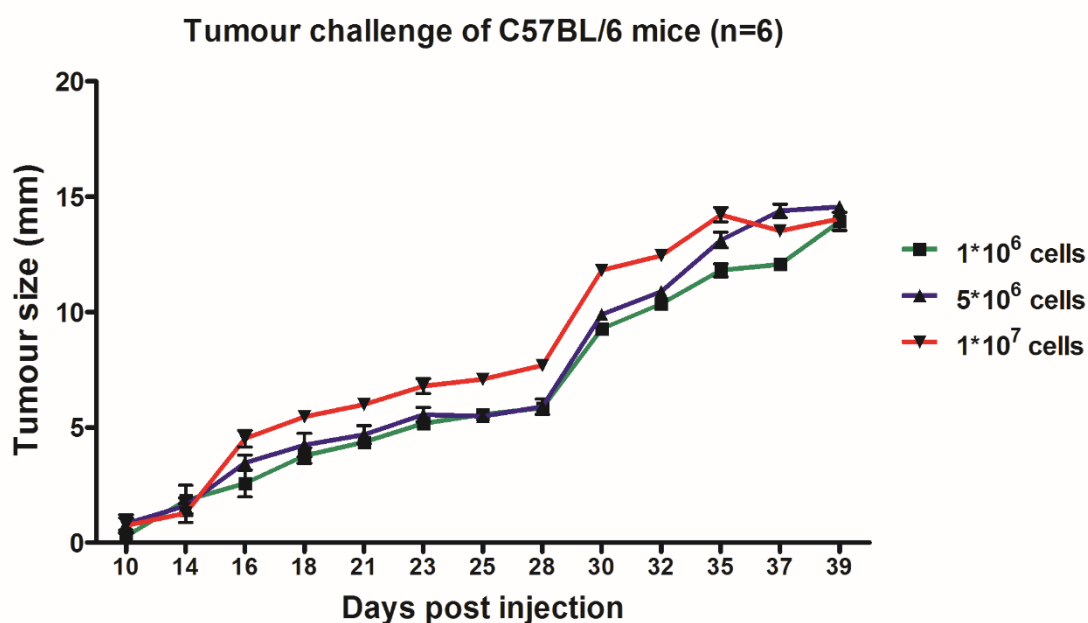
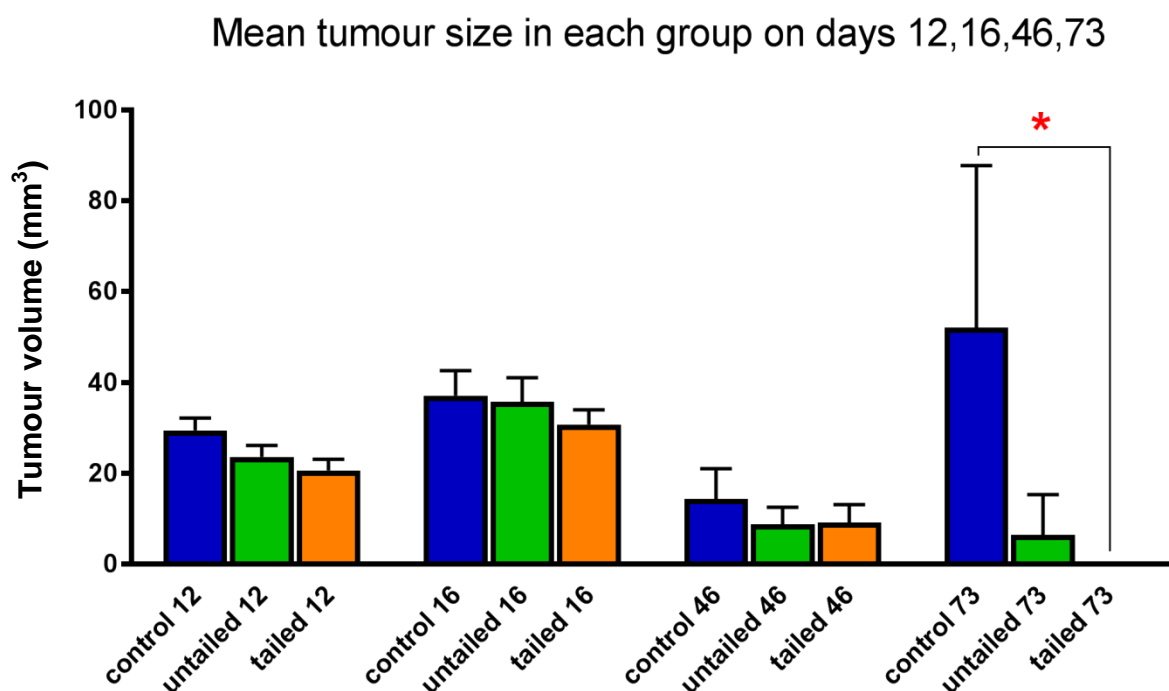


Figure 5.9: Dose response optimisation experiment for the *in vivo* tumour cell challenge. Six 6-8 weeks old C57BL/6 mice were subcutaneously injected with either  $1 \times 10^6$  total number of TRAMP-C2 cells, or  $5 \times 10^6$  or  $1 \times 10^7$  cells. There was no significant difference observed in the time or size of the tumour growth (n=6 /group,  $P > 0.05$ , 1-way ANOVA).

Following this experiment, the next step was to verify the *in vivo* activity of tailed and untailed compounds. Thirty 6-8 week old C57BL/6 mice were subcutaneously injected with  $5 \times 10^6$  TRAMP-C2 tumour cells in sterile PBS. At Day 12 post tumour injection, the animals were divided into three groups:

- Group 1 (control) was injected subcutaneously at the tumour site with sterile PBS;
- Group 2 was injected with untailed IL-15 and untailed murine antibodies to immune checkpoint inhibitors.
- Group 3 was injected with tailed IL-15 and the tailed PTL033 and PTL035

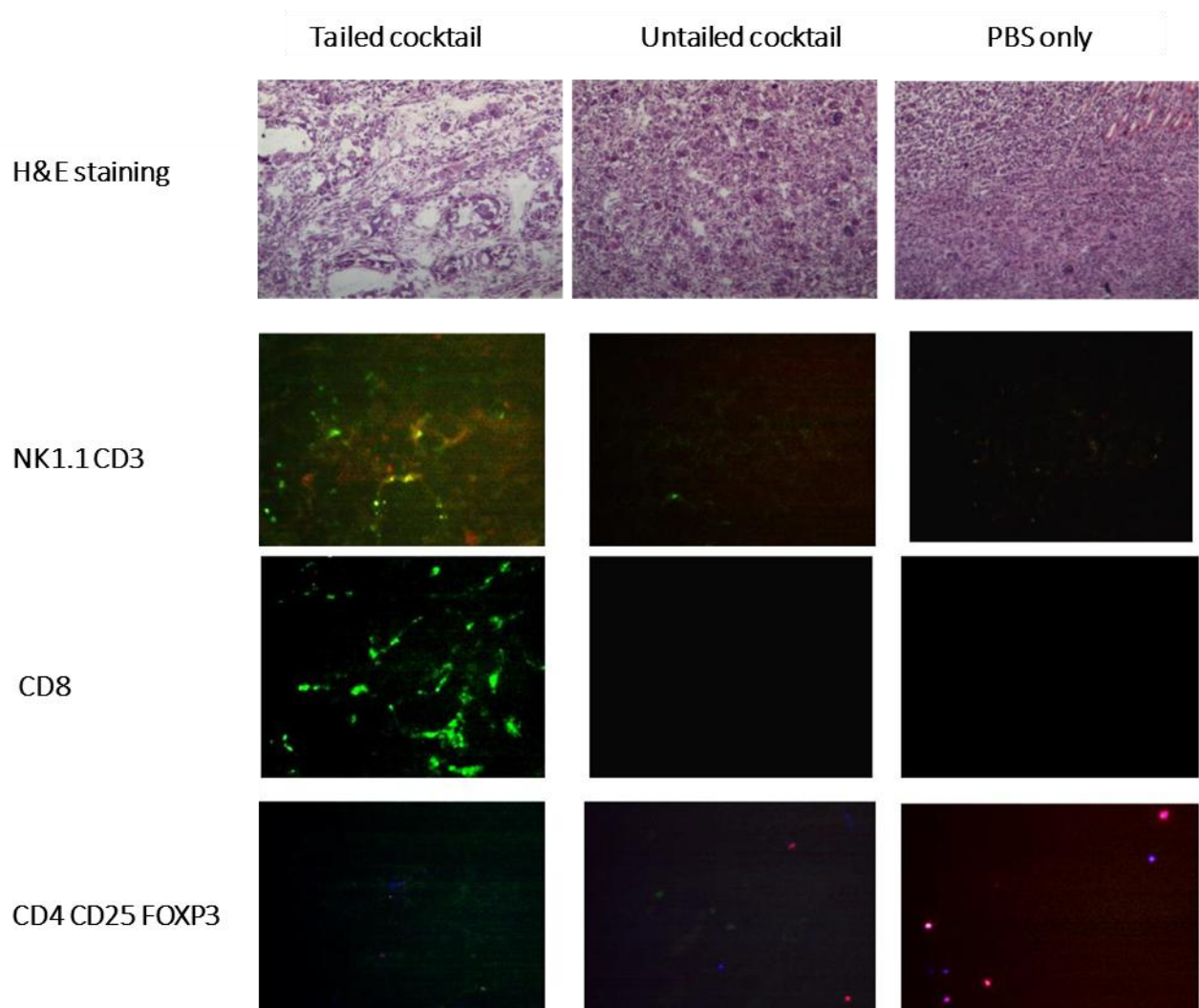
Tumour growth was measured up to 3 times per week for 73 days. A **complete clearance** of tumours by day 73 in the animals given just one injection of the cocktail of **tailed IL-15, PTL033 and PTL035** was observed (Figure 5.10).



**Figure 5.10: *In vivo* tumour cell challenge experiment.** Thirty 6-8 weeks C57BL/6 mice were subcutaneously injected with  $5 \times 10^6$  cells TRAMP-C2 cells in sterile PBS. At day 12 (D12) post tumour injection, the animals were divided in 3 groups; ten were injected with PBS at the site of the tumour (Group 1- control), ten with 5µg of untailed IL-15,100 µg PTL033 and 100 µg PTL035 (Group 2) and ten with the tailed version of the cocktail (Group 3). TRAMP-C2 tumour growth is shown to be completely cleared in the tailed cocktail group after 73 days whereas tumours were still present in mice injected with PBS or the untailed cocktail (n=10/group, \*p<0.05, 1-way ANOVA).

## 5.2.4 Histological analysis of the tumour tissue for the detection of infiltrating immune effector cells

Histological analysis of the tumour tissue obtained from the animals showed increased necrosis in the tailed injection group as seen with H and E staining and increased infiltration of NK cells and CD8 T cells compared to untailed and PBS groups (Figure 35).



**Figure 5.11: Histological analysis of tumour tissue.** Staining of tumours obtained from animals injected intra-tumourally with tailed cocktails of IL-15, PTL033 and PTL035, untailed cocktails of IL-15, PTL033 and PTL035 or PBS. Tissues were stained either with haematoxylin and eosin (H&E) or with antibodies to NK1.1, and CD3 (top panel Green and red respectively), CD8 (middle panel) or CD4, CD25 and FOXP3 (lower panel, Blue, green and red respectively). Infiltration of NK cells and CD8 T cells are pronounced in the tailed injection tumour group. Microscope used was Olympus BX51 microscope at x40 magnification.

### 5.3 Discussion

The *in vitro* coculture models, both allogeneic and syngeneic that were developed in our lab, have enabled us to test novel immunotherapeutics, using Dr Richard's Smith tailed protein agents. This protein modification technology, has been successfully used previously to create a tailed CR1 fragment (Mirococept), which is now on phase II kidney transplant trials (Patel, Smith *et al.* 2006).

The IL-15 protein was attached to a tail as observed by silver nitrate staining and western blots and flow cytometry confirmed that the tailed IL-15 was capable of binding to cell membranes. Thereafter, the *in vitro* activity of the tailed IL-15 protein was investigated through its ability to induce CTLL-2 cell proliferation- as measured by a previously described cell proliferation technique, MTS (Soman, Yang *et al.* 2009) - and through its ability to induce human and murine NK cell proliferation.

A fully functional tailed IL-15 form was produced; it induced the proliferation of the CTLL-2 cell line and of the murine and human NK cells, when compared to the control (no cytokine addition), or at similar or higher levels than the non-tailed form of IL-15. Furthermore, a lower concentration of tailed IL-15 was needed to observe the above effects. This cytotopic technology enables any protein of interest to become modified to attach to the cell membranes and exert its effect for a much prolonged period. Consequently, the finding that tailed IL-15 has either similar or better effects to the non-tailed IL-15, is a promising result, suggesting that the expansion of NK, NKT and CD8 T cells and the killing of the PCa cells could be much higher, if this protein is injected at the site of the tumour *in vivo*.

Two antibodies to immune checkpoint inhibitors were chosen to be part of a cocktail of drugs that were used further in an *in vivo* mouse model. These two antibodies were also attached to a tail and the cytotopic modification was confirmed by flow cytometry. The activity of the PTL033 was studied by measuring re-constitution of IL-2 secretion in the Jurkat cells, whereas that of the PTL035 was studied by prevention of binding of receptor and ligand on EL-4 cells, using flow cytometry. The results were very promising since equal or better activity of the tailed proteins was observed when compared to the non-tailed forms.

An *in vivo* tumour challenge model of C57BL/6 mice injected with TRAMP-C2 tumour cells was employed for the testing of the efficacy of the cocktail of tailed drugs (IL-15, PTL033, PTL035).

Before proceeding with this experiment though, a dose response experiment was performed, in order to find the optimal dose of injected TRAMP-C2 cells leading to the fastest tumour development. Various different cell concentrations have been previously used in literature on tumour cell challenge experiments (Kwon, Foster et al. 1999), however we chose to use a concentration of  $5 \times 10^6$  total numbers of cells, since all three different concentration of cells producing a similar result.

To assess the efficacy of the three tailed proteins, thirty C57BL/6 mice were subcutaneously injected with the optimised dose of TRAMP-C2 cells and at day 12 they were again injected subcutaneously at the site of the tumour with either PBS (control- group 1) or a cocktail of the three therapeutic agents- IL-15, PTL033 and PTL035 either in untailed (group 2) or tailed form (group 3). Our results showed that the tumours were cleared by day 73 in all mice injected with a single injection of the tailed cocktail, unlike the other two groups of mice. Nevertheless, the mice that were injected with the untailed cocktail of drugs also showed a regression in their tumours, which was significant when compared to the control group, but as the tailed proteins have the ability to attach to the cell membranes, they are also able to exert their functions for a prolonged period of time; hence in the group of mice that were injected with tailed drugs, the tumours had all disappeared.

In addition, tumour tissue was collected from each group of mice to test for the infiltration of immune effector cells. The H and E staining showed increased necrosis in the tailed injection group and the immunohistochemistry, showed increased NK and CD8 T cell infiltration in the tissue obtained from the tailed group of mice, when compared to the PBS group or the untailed group.

This result proved the unique efficacy of tailed IL-15 *in vivo* as well as *in vitro*, although the *in vivo* challenge with tailed IL-15 alone also needs to be performed.



## CHAPTER 6

### General Discussion and Future Work

## 6.1 Discussions and Conclusions

In this project we aimed to create *in vitro* and *in vivo* models of tumour- immune effector cell interaction and tumour cell killing which would enable us to identify, test and optimize novel immunotherapeutics. Our focus was to create non-cell based immunotherapies, such as cytokine treatments, which would be injected at the site of the tumour lesion, in order to overcome the immunosuppressive prostate tumour microenvironment, to enhance the efficacy of those and to avoid the toxic side effects that are observed upon systemic injection of a cytokine.

The *in vitro* creation of an allogeneic coculture model of immune effector cells and prostate cancer cell lines, both metastatic and non-metastatic, that was designed for these experiments, resembles the PCa microenvironment and it has enabled us to study the effects of a panel of therapeutic Th1 cytokines, in the immune effector cell proliferation and function. The cytokines studied included IL-2, IFN $\gamma$ , IL-12, IL-15 and IL-21.

Preliminary data by Mr. Oussama Elhage in our laboratory, has shown that in this allogeneic coculture system, IL-15 was the most potent activator of NK, NKT and CD8 T cells aiding the expansion of these cells and the killing of the PCa cell lines, when compared to the other therapeutic Th1 cytokines that were investigated (Elhage, Galustian *et al.* 2011). However, as there was only one coculture ratio of immune effector cells to PCa cells studied (8:1 E:T ratio), we aimed to investigate, whether other E:T ratios would produce different effects on the expansion of these immune effector cells and their cytotoxicity. In the literature there have been multiple E:T ratios tested (Marten, Renoth et al. 2001, Balsamo, Vermi et al. 2012, Shi, Fan et al. 2014), so in our allogeneic coculture system, both with PC-3 and LNCaP cell lines, three different ratios (8:1, 4:1, 2:1) were tested for the ability of IL-15 to expand NK, NKT and CD8 T cells (naïve, not primed) and increase the perforin and CD107a expression by these cells. Additionally, the optimal concentration of IL-15 (0.5-25ng/ml) that would produce the highest percentage PCa cell death by the immune effector cells was investigated, as well as the optimal coculture E:T ratio (those tested included 20:1, 8:1, 4:1, 2:1, 1:1, 0.5:1).

Taking both the phenotypic and the cell killing results into account, it was decided that the optimal ratio for coculturing the immune effector cells with the PCa cell lines (for both PC-3 and LNCaP) was 4:1 and the optimal concentration of IL-15 was 2.5ng/ml. Although the 8:1 coculture ratio had given similar results to the 4:1 ratio, and sometimes even better results when considering the CD107a expression by NK cells (in cocultures with PC-3 cell line), the PCa cell killing was considerably better at the 4:1 E:T ratio in cocultures with both cell lines. On the results presented in this thesis, although the CD8 T cells were expanded in the presence of IL-15 when the immune effector cells were cultured alone, in cocultures with PC-3 or LNCaP cell lines, IL-15 did not increase the expansion of CD8 T cells, unlike what was previously shown by Elhage *et al.* (Elhage, Galustian *et al.* 2012). This difference may be due to the fact that in the preliminary experiments the concentration of IL-15 that was used was 25ng/ml, whereas in the experiments of this thesis, the concentration used was 2.5ng/ml. It was shown by Ms Fiona Watkinson from the Protein Therapeutics Laboratory, that the highest expansion of NK cells is observed in the presence of 2.5ng/ml IL-15, and at concentrations above that, the NK cells expand less. It may be that CD8 T cells need a higher concentration of IL-15 to expand rather than 2.5ng/ml that was used for our work, hence the discrepancy found between the preliminary and this thesis' results. Furthermore, it was also shown by Elhage *et al.* in a separate study investigating the effects of depleting CD56<sup>+</sup> cells and CD8 T cells on IL-15 mediated effector-cell induced PCa cell killing and expansion of CD8 and CD56 cells respectively within the cocultures, CD8 T cell depletion enhanced the NK cell mediated PCa cell killing, suggesting that CD8 T cells may hinder the CD56<sup>+</sup> cell cytotoxic activity (Elhage, Sakellariou *et al.* 2015). Hence our finding that IL-15 does not expand the CD8 T cells in cocultures of immune effector cells with PC-3 or LNCaP cell lines, does not affect the PCa cell killing which is attributed to NK cells.

Following the optimization of the allogeneic coculture model's E:T ratio, a syngeneic coculture model was set up, in order to investigate whether alloreactivity is aiding the expansion of NK and NKT cells. The syngeneic coculture model was comprised of murine NK- or T- enriched cells derived from the spleen of a C57BL/6 mouse, cocultured with the TRAMP-C1 cell line; a PCa cell line also derived from the transgenic adenocarcinoma of the C57BL/6 mouse prostate. Similar to the allogeneic model, E:T ratios were investigated; IL-15 significantly increased the killing of the TRAMP-C1 cells by the NK cells at E:T ratios of 1:1, 2:1

and 4:1. However, taking into account that the highest NK cell expansion by IL-15 was observed at 4:1 E:T ratio, it was decided to use that ratio for further experiments. As a control measurement, it was investigated, whether IL-15 was toxic towards the TRAMP-C1 cells, without addition of the immune effector cells. So, TRAMP-C1 cells were cultured alone at different cell concentrations with or without the addition of IL-15: it was observed that IL-15 did not enhance the TRAMP-C1 killing, confirming that it modulates only the immune effector cell proliferation and function of these in coculture models.

NK- or T- enriched cells were cocultured in the presence or absence of TRAMP-C1 cells, in the presence or absence of a panel of therapeutic Th1 cytokines (i.e IL-2, IL-12, IL-15, IL-21, IFN $\gamma$ ) as the allogeneic coculture model was designed. It was found that indeed IL-15 was the only cytokine that highly expanded the NK, NKT and CD8 T cells, when compared to the control (no cytokine) or to the other Th1 cytokines. IL-15 did not affect the T regulatory cells, as it was also shown in the preliminary data results from the allogeneic coculture model.

IL-15 has been previously shown to enhance NK and T cell proliferation (Fehniger, Suzuki *et al.* 2001); (Gravisaco, Mongini *et al.* 2003, Sato, Sabzevari *et al.* 2011). However, to our knowledge we are the first group in the UK, to show that IL-15 is also active when effector immune cells are cultured in the presence of PCa cell lines.

Based on the above observations, and as the NK cells bear receptors on their surface that regulate the killing of the target cells, it was investigated whether the IL-15 mechanism of action is through the modulation of inhibitory, activatory and natural cytotoxicity receptors on NK cells and their associated ligands on the PCa cells.

NK cells bear a number of aNKRs and iNKRs on their surface that enable them to modulate immune responses and maintain tissue homeostasis. The cytolytic activity of NK cells is regulated by positive signalling of the aNKRs which trigger cell lysis, and by negative signalling of the iNKRs that prevent cell lysis. In this study, we aimed to investigate how IL-15 modulates the ability of NK cells to kill PCa cells and therefore we investigated the IL-15 effects on three KIR receptors (KIR3DL1, KIR2DL1, KIR2DL2) and on five aNKRs (DNAM-1, NKG2D, NKp30, NKp44, NKp46), using the allogeneic coculture model and observing the percentage expression of each receptor by the NK cells.

Physiologically, low amounts of IL-2 should be present in the prostate; therefore we aimed to mimic the prostate microenvironment, by studying the effects of IL-15 in the presence of low concentration of IL-2, apart from IL-15 alone.

Initially the effects of IL-2 and IL-15 on the receptor expression by NK cells, when PBMCs are cultured alone, were investigated and it was found that IL-2 did not have any significant effect on any of the receptor expression when compared to the control (no cytokine), whereas IL-15 highly upregulated KIR2DL2, NKp46 and DNAM-1 expression. However, in the presence of the PC-3 cell line, IL-15 did not seem to affect the expression of any of these receptors that it upregulated on PBMCs, but it only highly upregulated the expression of NKG2D. In the presence of LNCaP cells though, IL-15 still upregulated the KIR2DL2 expression as well as that of DNAM-1 and it downregulated the expression of NKp30.

Our finding that in cocultures of immune effector cells with PC-3 cells, IL-15 highly increases the NKG2D expression, suggests that IL-15 increases the NK cytotoxicity in an NKG2D dependent- manner. The initial coculture data of immune effector cells with LNCaP cells (figure 4.10) did not show a significantly increased NKG2D expression by NK cells in the presence of IL-15, although when these experiments were repeated in figure 4.20, IL-15 seemed to highly significantly upregulate NKG2D. This discrepancy may be due to donor to donor differences in the amount of NK cells and of the NKG2D per donor; the standard deviation in the first set of experiments is much larger when compared to the second set of experiments; hence it is assumed that variation in donor NK/NKG2D percentages gave a non-significant result.

The result of the second experiment (figure 4.20), where NKG2D expression is highly upregulated in the presence of IL-15, in cocultures with LNCaP cells, is in compliance with other published studies, where IL-15 has been shown, in two separate studies, to increase the NKG2D receptor in acute myeloid leukaemia patients with a concomitant increase in NK cytotoxicity against autologous leukemic blasts (Szczepanski, Szajnik *et al.* 2010) (Anguille, Van Acker *et al.* 2015). Additionally, other studies have suggested that IL-15 regulates the cytotoxic T lymphocytes' activity by regulating the NKG2D pathway in an inflammatory environment, as in the case of tuberculosis (Tagaya, Bamford *et al.* 1996, Roberts, Lee *et al.* 2001, Rausch, Hessmann *et al.* 2006).

The increased expression of KIR2DL2 and of DNAM-1 by IL-15 is also in agreement with existing literature, which suggests that IL-15 increases the expression of DNAM-1 (Hromadnikova, Li *et al.* 2016) as well as NKp30, NKp44 and NKp46 (de Rham, Ferrari-Lacraz *et al.* 2007), with a concomitant increase in the NK cytotoxicity.

A decrease in the expression of natural cytotoxicity receptors would be expected to result in a decreased activity of the NK cells; NK cells are activated to kill both PC-3 and LNCaP cells in the presence of IL-15 and therefore the significance of the decreased expression of NKp30 with LNCaP and not PC-3 cells is not clear. Due to the complexity of the above controversial findings and also due to contradicting literature on the effects of cytokines on these receptors, we went on to examine the corresponding ligands of these NKRs on PCa cells.

The surface expression of the NKR ligands by the PC-3 or LNCaP cell lines was not significantly affected by the presence of IL-2 or of IL-15. However in cocultures of non-adherent PBMCs and PC-3 cells, the presence of IL-15 significantly downregulated the expression of ULBP1 (a ligand for the NKG2D receptor), when compared to the effects of IL-2 or of the control. In the case of cocultures with the LNCaP cell line, IL-2 did not have any effect on any of the ligands, whereas IL-15 significantly downregulated MICA and ULBP1 (ligands for NKG2D), Nectin-2 (ligand for DNAM-1) and HLA-I Bw4 (ligand for KIR3DL1).

IL-15 may be aiding the cytotoxic capacity of NK cells towards the LNCaP cells by increasing the expression of DNAM-1 in NK cells and decreasing the corresponding Nectin-2 ligand on LNCaP cells. Kritikou *et al* (2016) also suggested that increased DNAM-1 expression could lead to an increased tumour cell killing of cells that express that ligand, as was observed on B16 melanoma cells and RMA-S lymphoma cells (Kritikou, Dahlberg *et al.* 2016).

The effect of IL-15 on KIR receptors and on their corresponding ligands is a poorly understood concept and there are limited or no previously published studies suggesting a putative effect of IL-15. It is important to note though, that NK cell cytotoxicity is triggered by the loss of MHC Class I on tumour cells (Moretta and Moretta 2004, Bachanova and Miller 2014), so possibly the high downregulation of HLA-I Bw4 by IL-15, signifies the increased cytotoxic activity of NK cells towards the PCa cells.

Although the relationship between IL-15 and ULBP1 is another poorly researched area, a study by Sutherland *et al.* (2006) supports our theory, that IL-15 enhances the NK cytotoxicity towards the PCa cells by downregulating the ULBP1 ligand, as they have shown that in

C57BL/6 SCID mice that were challenged with EL-4 cells expressing the ULBP1 ligand, IL-15 enhanced the tumour rejection, compared to the mice that were EL-4 control cells (Sutherland, Rabinovich *et al.* 2006).

The effects of the NKG2D - MICA interaction in cancer have been at the centre of investigation for many years, resulting in ambiguous opinions (Zhang, Basher *et al.* 2015). In this project, we investigated this interaction in regards to IL-15 and aimed to understand what the mechanism of action of IL-15 is, as it is shown that IL-15 enhances the NK cell expansion and its cytotoxic activity towards the PCa cells in our allogeneic coculture model. Apart from investigating the expression of NKG2D and its ligand MICA at the surface of NK and PCa cells respectively, we investigated the concept of shedding of the ligand in a soluble form. It has been suggested that shedding of MICA by tumour cells promotes the tumour immune evasion (Groh, Wu *et al.* 2002, Doubrovina, Doubrovin *et al.* 2003, Raffaghello, Prigione *et al.* 2004, Wu, Higgins *et al.* 2004).

Using the allogeneic coculture model of immune effector cells with PC-3 or LNCaP cells, we found that IL-15 a) increases the NKG2D surface expression in both coculture models, b) decreases substantially the MICA surface expression in the cocultures with LNCaP and when investigating the concept of shedding of this ligand in the supernatants of the cocultures of immune effector cells with PC-3 or LNCaP cells, and c) highly downregulates the soluble expression of MICA when compared to the control. Unfortunately, blocking of MICA in our coculture model was not successful, however blocking of NKG2D did not seem to affect the cytotoxicity of NK cells towards the PCa cells. A study that was published this year by Wu (2016) suggests that neutralizing the soluble sMIC in a humanized model of metastatic PCa, significantly reduced the prostate tumours and prevented metastasis (Wu 2016).

Our study therefore proposes that IL-15 enhances the NK cytotoxicity towards the PCa cells in a coculture model of immune effector cells with PCa cell lines, which has been optimised to mimic the PCa microenvironment, by upregulating the NKG2D receptor expression and downregulating the surface and soluble expression of its ligand MICA.

Following the investigation of the mechanism of action of IL-15 we assessed its activity *in vivo*, as part of a therapeutic cocktail in C57BL/6 mice, challenged with TRAMP-C2

carcinoma cell line. IL-15 in combination with two antibodies to immune checkpoints (PTL033 and PTL036) underwent a protein modification, using a technology developed in Dr Richard Smith's laboratory called cytotopic modification; by this, the protein of interest is attached to a 'tail' molecule consisting of a myristoyl group and a cationic peptide sequence terminated with an activated thiol group that can link to free cysteines in the protein of interest (Smith and Smith 2001). Applying this technology to immunotherapeutic proteins, enables them to be localised within the tumour lesions, breaking the tolerance within this immunosuppressive microenvironment, avoiding the systemic toxicity of these agents and inducing a potent immune response, even when less of these agents is used.

Dr Dorota Smolarek from the Protein Therapeutics Laboratory cytotopically modified the IL-15 and the two antibodies to immune checkpoints, which comprised the cocktail of drugs that was tested *in vivo*. Prior to that though, the activity of the tailed cocktail of drugs was tested using *in vitro* assays. The activity of tailed IL-15 and of PTL033 and PTL035 was shown to be either equal or better to the non-tailed forms of these agents, hence we proceeded with the *in vivo* tumour challenge experiment: C57BL/6 mice were subcutaneously injected with the TRAMP-C2 cell line and at day 12 they were again subcutaneously injected as close to the tumour as possible, either with PBS, or non-tailed forms of the cocktail of drugs or with the tailed forms of the cocktail. The growth of the tumour in the mice was monitored every one or two days, and by day 73 the mice injected with the tailed forms of the cocktail of drugs showed complete clearance of their tumours, unlike the other two groups of mice injected with PBS or untail cocktail.

Tumour tissue was collected from each mouse in every group that grew a tumour and the tissue was stained with H&E or for immune cell infiltration of NK, CD8 T and Treg cells. Increased tissue necrosis was shown by H&E on the tissue obtained from the tailed group, as well as increased NK and CD8 T cell infiltration, when compared to the other groups.

Overall in this thesis, we have identified a cytokine, IL-15, that seems to be the most potent activator of NK, NKT and CD8 T cells, aiding their expansion and cytotoxic activity towards the PCa cells in an *in vitro* allogeneic coculture model of immune effector cells with PCa cells, which mimics the PCa immunosuppressive microenvironment. The mechanism by which IL-15 aids the cytotoxic activity of NK cells towards the PCa cells seems to be through the upregulation of the NKG2D receptor on the surface of NK cells, with a concomitant



downregulation of its ligand MICA both on the surface of the PCa cells and in a soluble form. When tested *in vivo*, a cocktail of IL-15 with two antibodies to immune checkpoints, that have been biochemically modified to localise at cell membranes, so called 'tailed', also seem to prove more efficacious, when compared to their non-tailed forms or control, as they completely clear the tumours from mice, only with a single injection of the tailed cocktail at the tumour lesion.

## **6.2 Future work**

The readout for NK cell expansion has been mainly based on the measurement of the CD56 percent expression within the population of non-adherent PBMCs in the human coculture model. However, it is reported that CD56 dim and bright populations have different functions, hence it would be reasonable to examine these in our human coculture models. We should also examine the effects and function of the CD16 dim and bright NK cells. Additional readouts for effector cell expansion for NK, NKT and CD8 T cells in the human coculture model and within the splenocytes enriched for NK cells or CD8 T cells should include the CFSE (Carboxyfluorescein succinimidyl ester) incorporation and Ki67 expression; both are dyes that marker cell proliferation.

NK receptor and ligand experiments were performed using the *in vitro* allogeneic coculture model of non-adherent PBMCs with PCa cell lines. However in order to exclude any results that are attributed to alloreactivity in our model, these experiments should also be performed using the *in vitro* syngeneic coculture model.

Although the proposed mechanism of action of IL-15 in enhancing the NK cell expansion and aiding its cytotoxicity towards the PCa cell lines, was suggested to be through the upregulation of the NKG2D receptor on NK cells with a concomitant decrease in the MICA surface and soluble expression on the PCa cell lines, MICA was not successfully blocked in our system; hence this should be further investigated, potentially using an even higher concentration of the blocking aMICA antibody and even by the purchase of a different neutralising antibody. Furthermore, it would be beneficial to investigate whether HLA-I Bw4, a NKR ligand that was highly downregulated by IL-15 provides an additional enhancement on the IL-15 activity.

The *in vivo* challenge experiment was only performed once, although 10 animals were used in each group giving a strongly significant result and should be repeated in the future. Unfortunately the time was a limiting factor from repeating the experiment more times since different variables had to be optimised (e.g. Needle size, specific C57BL/6 mouse strain) in order to achieve a desirable tumour size. Perhaps using a different tumour cell line, such as

RM9, a rapidly growing prostate tumour cell line, could provide us with a more potent mouse tumour challenge.

The challenge model should also be repeated using the individual components of the therapeutic cocktail in tailed and non-tailed form. Yu et al. (Yu, Steel et al. 2012) found that IL-15 alone did give a 50% reduction of tumour volume.

Based on the proposed mechanism of action of IL-15, a next step should also be to tumour-challenge mice that are NKG2D knockouts and examine the effects of our tailed and non-tailed cocktail of drugs on these.

## Appendix

PC-3 cells were cultured alone, in the presence or absence of IL-2 (1ng/ml or 10ng/ml) or of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml), in order to observe the levels of the ligand expression by this cell line and whether these are altered in the presence of IL-2 or IL-15 cytokines. No significant change in any of the ligands' expression by PC-3 cells was observed in the presence of cytokines, when compared to control (no cytokine addition) (n=3-5,  $p>0.05$ , 1-way ANOVA) (Figure A.1-4).

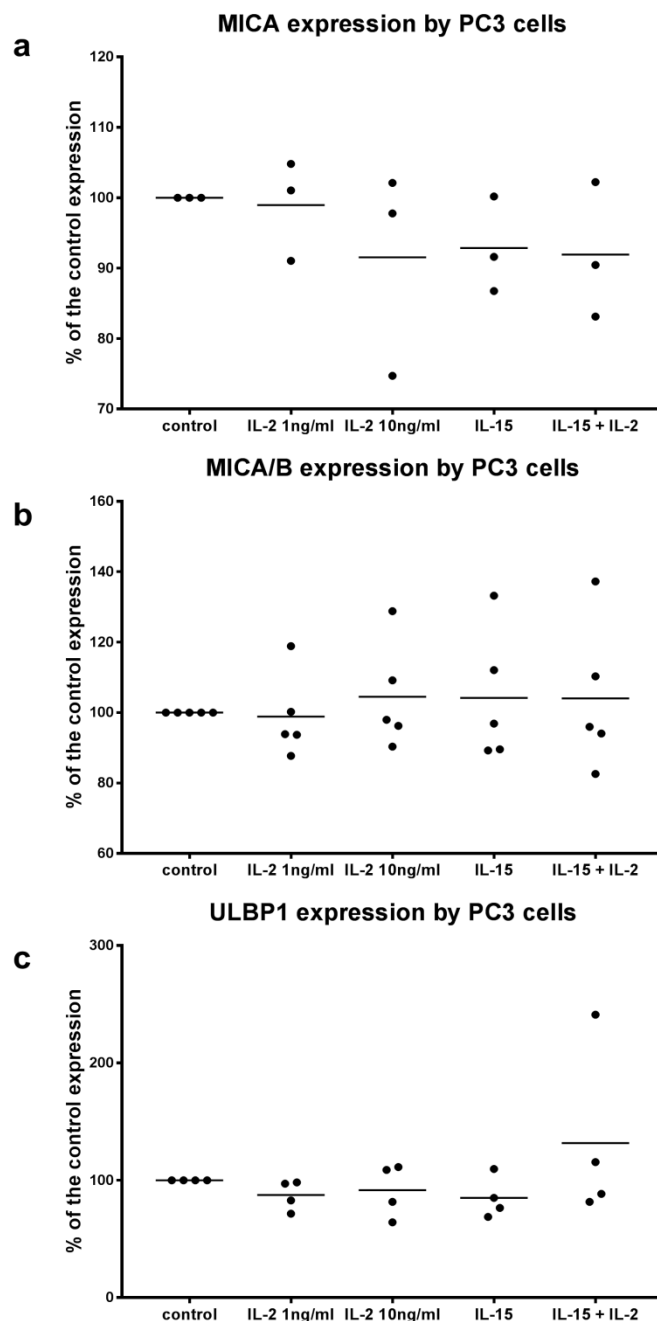


Figure A.1 a-c: Expression of the NKG2D ligands, MICA, MICA/B and ULBP1 by PC-3 cells. PC-3 cells were cultured in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml)(n=3-5,  $p>0.05$ , 1-way ANOVA).

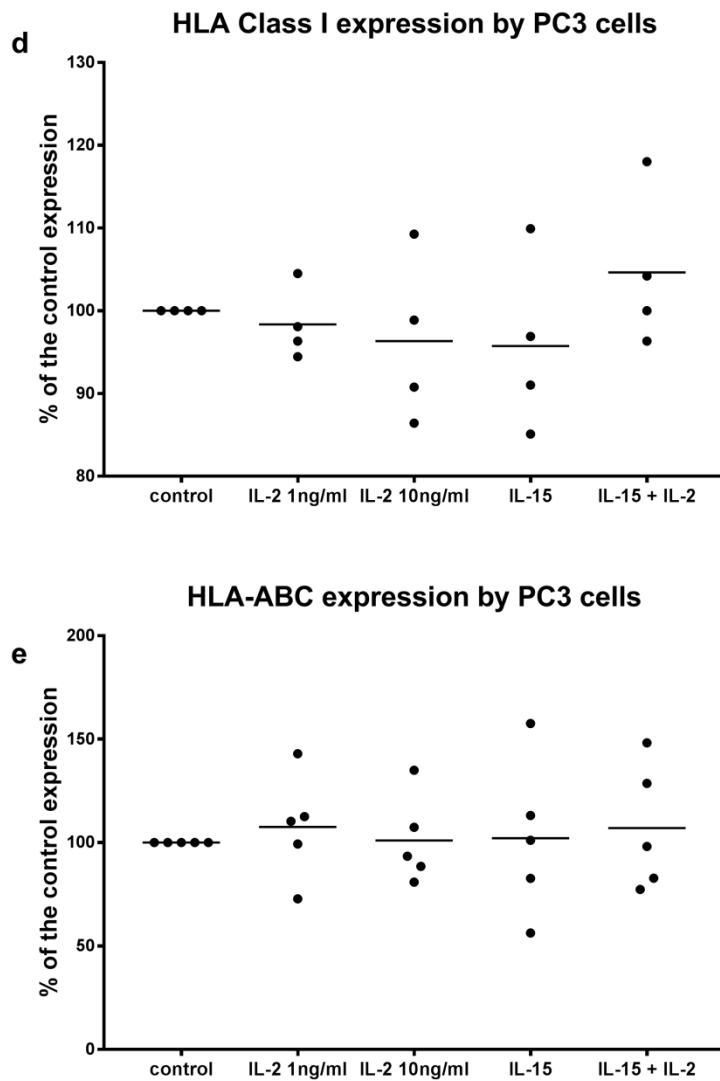


Figure A.2 d,e : Expression of HLA Class I and HLA-ABC by PC-3 cells. PC-3 cells were cultured in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=4-5,  $p>0.05$ , 1-way ANOVA).

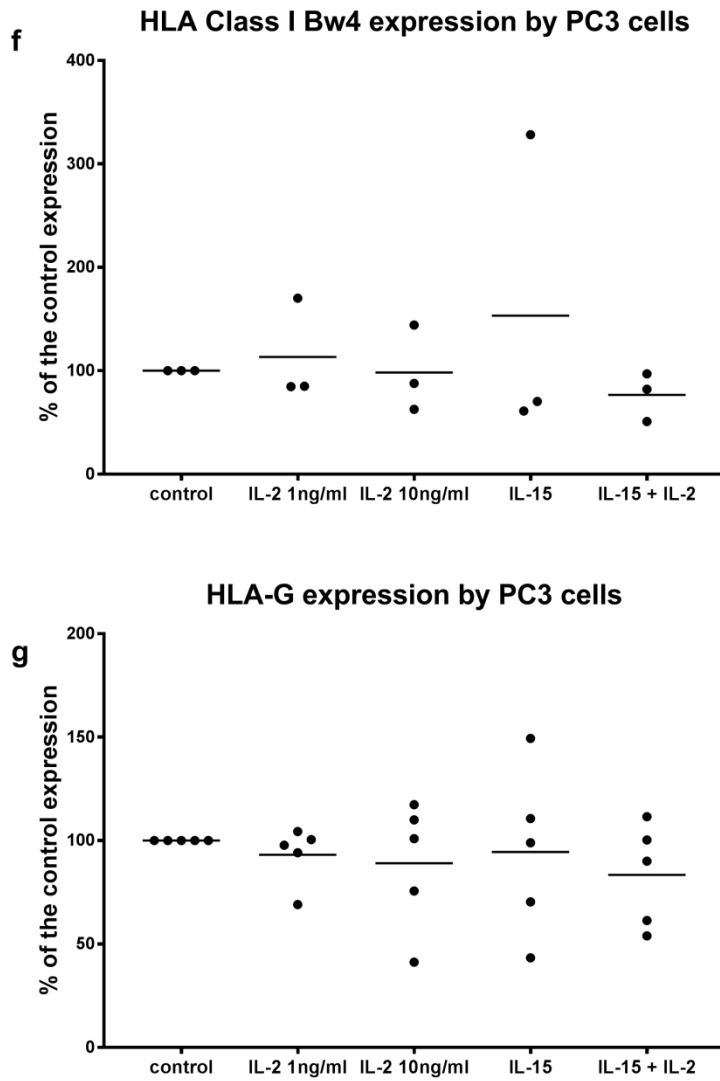


Figure A.3 f,g : Expression of HLA Class I Bw4 and HLA-G by PC-3 cells. PC-3 cells were cultured in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=3-5,  $p>0.05$ , 1-way ANOVA).

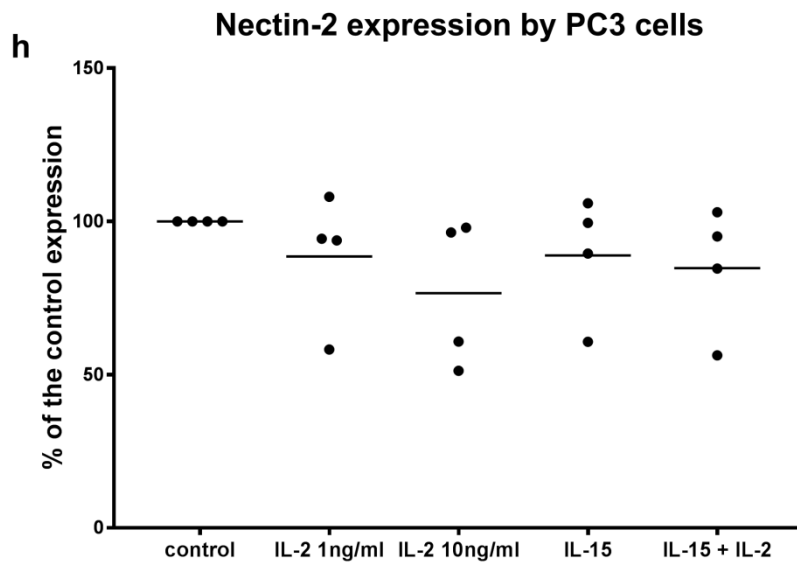


Figure A.4 h: Expression of the DNAM-1 ligand, Nectin-2, by PC-3 cells. PC-3 cells were cultured in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml)(n=4,  $p>0.05$ , 1-way ANOVA).



LNCaP cells were cultured alone, in the presence or absence of IL-2 (1ng/ml or 10ng/ml) or of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml), in order to observe the levels of the ligand expression by this cell line and whether these are altered in the presence of IL-2 or IL-15 cytokines. No significant change in any of the ligands' expression by LNCaP cells was observed in the presence of cytokines, when compared to control (no cytokine addition) (n=4, p>0.05, 1-way ANOVA) (Figure A.5-8).

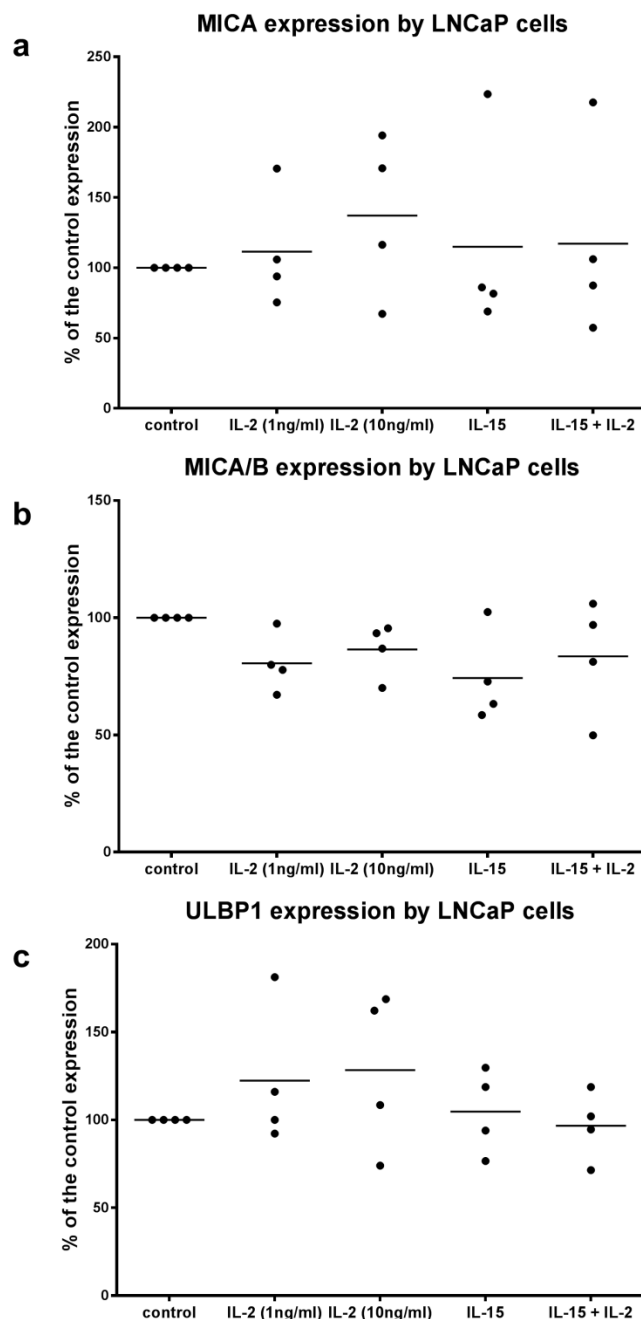


Figure A.5 a-c : Expression of the NKG2D ligands, MICA, MICA/B and ULBP1 by LNCaP cells. LNCaP cells were cultured in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=4, p>0.05, 1-way ANOVA).

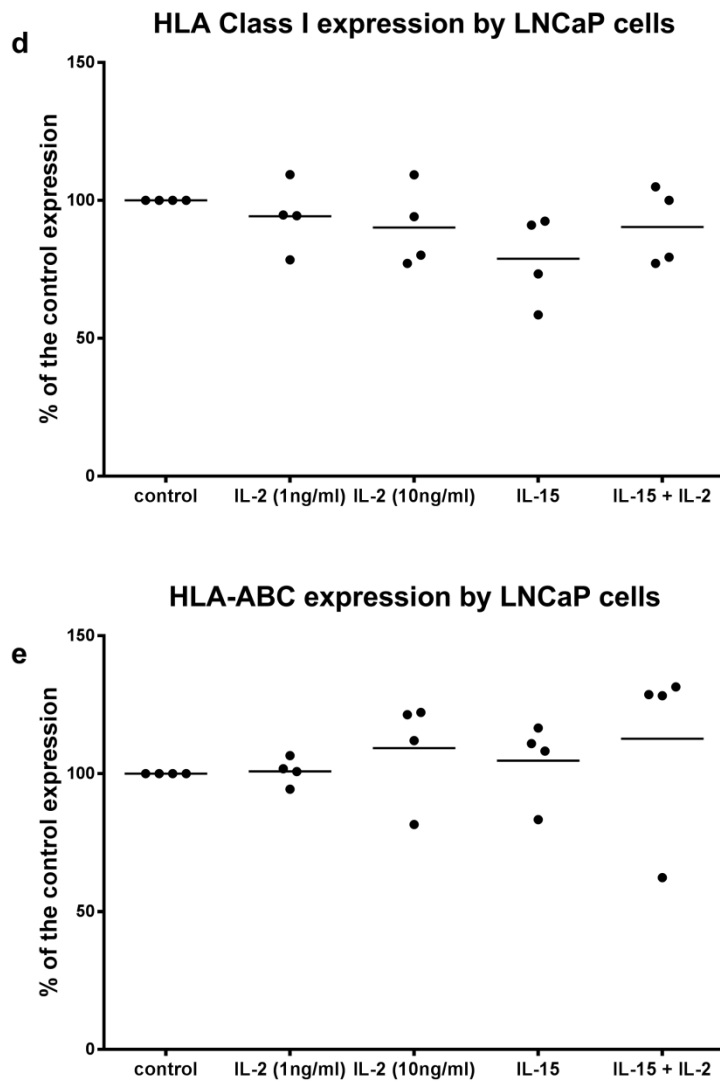


Figure A.6 d,e : Expression of HLA Class I and HLA-ABC by LNCaP cells. LNCaP cells were cultured in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=4, p>0.05, 1-way ANOVA).

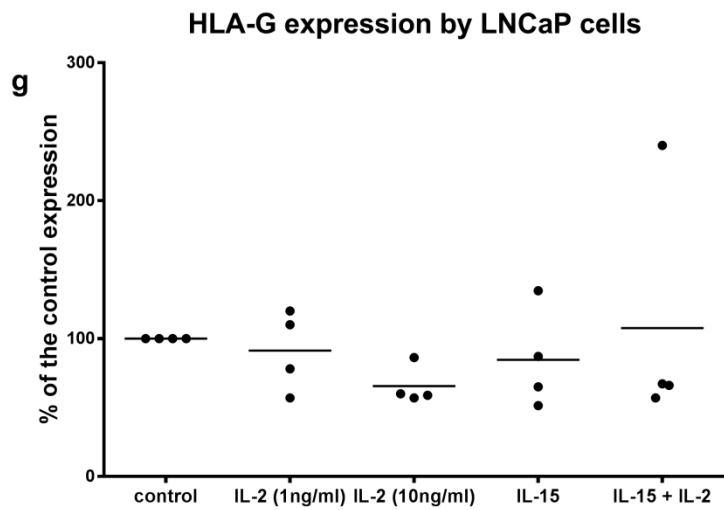
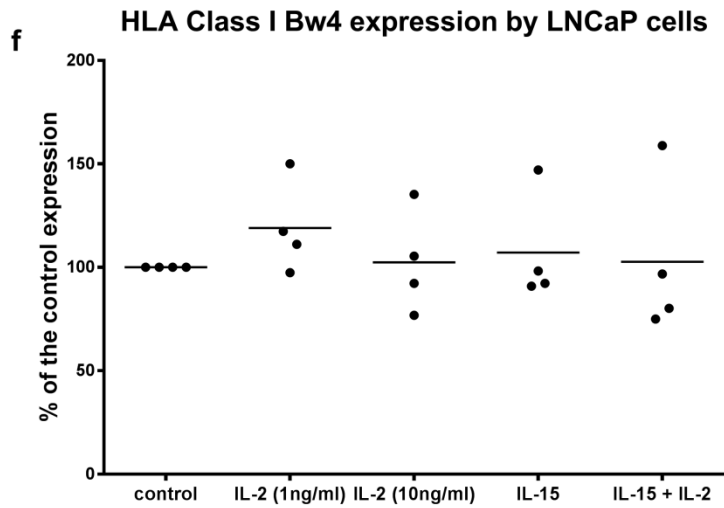


Figure A.7 f,g : Expression of HLA Class I Bw4 and HLA-G by LNCaP cells. LNCaP cells were cultured in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=4,  $p>0.05$ , 1-way ANOVA).

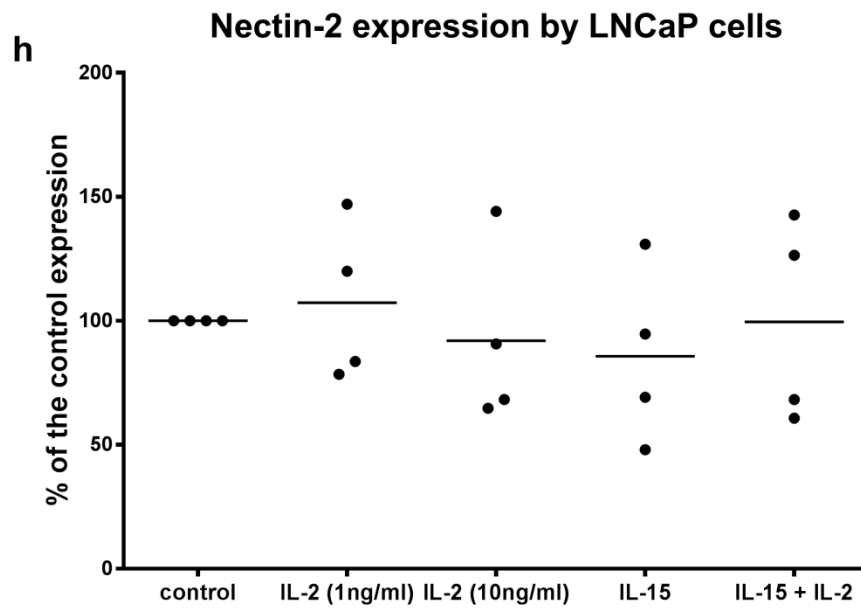


Figure A.8 h : Expression of the DNAM-1 ligand, Nectin-2, by LNCaP cells. LNCaP cells were cultured in the presence or absence of IL-2 (1 or 10ng/ml) and of IL-15 (2.5ng/ml) with or without IL-2 (1ng/ml) (n=4, p>0.05, 1-way ANOVA).

## References

- Agata, Y., A. Kawasaki, H. Nishimura, Y. Ishida, T. Tsubata, H. Yagita and T. Honjo (1996). "Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes." Int Immunol **8**(5): 765-772.
- Ahuja, S. S., F. Paliogianni, H. Yamada, J. E. Balow and D. T. Boumpas (1993). "Effect of transforming growth factor-beta on early and late activation events in human T cells." J Immunol **150**(8 Pt 1): 3109-3118.
- Allez, M., V. Tieng, A. Nakazawa, X. Treton, V. Pacault, N. Dulphy, S. Caillat-Zucman, P. Paul, J. M. Gornet, C. Douay, S. Ravet, R. Tamouza, D. Charron, M. Lemann, L. Mayer and A. Toubert (2007). "CD4+NKG2D+ T cells in Crohn's disease mediate inflammatory and cytotoxic responses through MICA interactions." Gastroenterology **132**(7): 2346-2358.
- Almand, B., J. I. Clark, E. Nikitina, J. van Beynen, N. R. English, S. C. Knight, D. P. Carbone and D. I. Gabrilovich (2001). "Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer." J Immunol **166**(1): 678-689.
- Anderson, D. M., S. Kumaki, M. Ahdieh, J. Bertles, M. Tometsko, A. Loomis, J. Giri, N. G. Copeland, D. J. Gilbert, N. A. Jenkins and et al. (1995). "Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of IL15RA and IL2RA genes." J Biol Chem **270**(50): 29862-29869.
- Anguille, S., H. H. Van Acker, J. Van den Bergh, Y. Willemsen, H. Goossens, V. F. Van Tendeloo, E. L. Smits, Z. N. Berneman and E. Lion (2015). "Interleukin-15 Dendritic Cells Harness NK Cell Cytotoxic Effector Function in a Contact- and IL-15-Dependent Manner." PLoS One **10**(5): e0123340.
- Arigami, T., N. Narita, R. Mizuno, L. Nguyen, X. Ye, A. Chung, A. E. Giuliano and D. S. Hoon (2010). "B7-h3 ligand expression by primary breast cancer and associated with regional nodal metastasis." Ann Surg **252**(6): 1044-1051.
- Arigami, T., Y. Uenosono, M. Hirata, T. Hagihara, S. Yanagita, S. Ishigami and S. Natsugoe (2010). "Expression of B7-H4 in blood of patients with gastric cancer predicts tumor progression and prognosis." J Surg Oncol **102**(7): 748-752.
- Arruvito, L., F. Payaslian, P. Baz, A. Podhorzer, A. Billordo, J. Pandolfi, G. Semeniuk, E. Arribalzaga and L. Fainboim (2014). "Identification and clinical relevance of naturally occurring human CD8+HLA-DR+ regulatory T cells." J Immunol **193**(9): 4469-4476.
- Arteaga, C. L., S. D. Hurd, A. R. Winnier, M. D. Johnson, B. M. Fendly and J. T. Forbes (1993). "Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression." J Clin Invest **92**(6): 2569-2576.
- Atkins, M. B., L. Kunkel, M. Sznol and S. A. Rosenberg (2000). "High-dose recombinant interleukin-2 therapy in patients with metastatic melanoma: long-term survival update." Cancer J Sci Am **6 Suppl 1**: S11-14.
- Bachanova, V. and J. S. Miller (2014). "NK cells in therapy of cancer." Crit Rev Oncog **19**(1-2): 133-141.

Bachelet, I., A. Munitz, D. Mankutad and F. Levi-Schaffer (2006). "Mast cell costimulation by CD226/CD112 (DNAM-1/Nectin-2): a novel interface in the allergic process." J Biol Chem **281**(37): 27190-27196.

Balsamo, M., W. Vermi, M. Parodi, G. Pietra, C. Manzini, P. Queirolo, S. Lonardi, R. Augugliaro, A. Moretta, F. Facchetti, L. Moretta, M. C. Mingari and M. Vitale (2012). "Melanoma cells become resistant to NK-cell-mediated killing when exposed to NK-cell numbers compatible with NK-cell infiltration in the tumor." Eur J Immunol **42**(7): 1833-1842.

Barron, D. A. and D. R. Rowley (2012). "The reactive stroma microenvironment and prostate cancer progression." Endocr Relat Cancer **19**(6): R187-204.

Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier and T. Spies (1999). "Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA." Science **285**(5428): 727-729.

Belardelli, F., M. Ferrantini, E. Proietti and J. M. Kirkwood (2002). "Interferon-alpha in tumor immunity and immunotherapy." Cytokine Growth Factor Rev **13**(2): 119-134.

Beldegrun, A. S., T. Klatte, B. Shuch, J. C. LaRochelle, D. C. Miller, J. W. Said, S. B. Riggs, N. Zomorodian, F. F. Kabbinavar, J. B. Dekernion and A. J. Pantuck (2008). "Cancer-specific survival outcomes among patients treated during the cytokine era of kidney cancer (1989-2005): a benchmark for emerging targeted cancer therapies." Cancer **113**(9): 2457-2463.

Berke, G. (1994). "The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects." Annu Rev Immunol **12**: 735-773.

Bernardini, G., A. Gismondi and A. Santoni (2012). "Chemokines and NK cells: regulators of development, trafficking and functions." Immunol Lett **145**(1-2): 39-46.

Binstadt, B. A., K. M. Brumbaugh, C. J. Dick, A. M. Scharenberg, B. L. Williams, M. Colonna, L. L. Lanier, J. P. Kinet, R. T. Abraham and P. J. Leibson (1996). "Sequential involvement of Lck and SHP-1 with MHC-recognizing receptors on NK cells inhibits FcR-initiated tyrosine kinase activation." Immunity **5**(6): 629-638.

Bishop, J. L., A. Sio, A. Angeles, M. E. Roberts, A. A. Azad, K. N. Chi and A. Zoubeidi (2015). "PD-L1 is highly expressed in Enzalutamide resistant prostate cancer." Oncotarget **6**(1): 234-242.

Blank, C., I. Brown, A. C. Peterson, M. Spiotto, Y. Iwai, T. Honjo and T. F. Gajewski (2004). "PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells." Cancer Res **64**(3): 1140-1145.

Bottino, C., R. Castriconi, L. Moretta and A. Moretta (2005). "Cellular ligands of activating NK receptors." Trends Immunol **26**(4): 221-226.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-254.

Bronte, V., T. Kasic, G. Gri, K. Gallana, G. Borsellino, I. Marigo, L. Battistini, M. Iafrate, T. Prayer-Galetti, F. Pagano and A. Viola (2005). "Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers." J Exp Med **201**(8): 1257-1268.

- Bryceson, Y. T., M. E. March, H. G. Ljunggren and E. O. Long (2006). "Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion." Blood **107**(1): 159-166.
- Cardell, S., S. Tangri, S. Chan, M. Kronenberg, C. Benoist and D. Mathis (1995). "CD1-restricted CD4+ T cells in major histocompatibility complex class II-deficient mice." J Exp Med **182**(4): 993-1004.
- Carson, W. E., J. G. Giri, M. J. Lindemann, M. L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein and M. A. Caligiuri (1994). "Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor." J Exp Med **180**(4): 1395-1403.
- Cerwenka, A. and L. L. Lanier (2001). "Natural killer cells, viruses and cancer." Nat Rev Immunol **1**(1): 41-49.
- Chavin, G., Y. Sheinin, P. L. Crispen, S. A. Boorjian, T. J. Roth, L. Rangel, M. L. Blute, T. J. Sebo, D. J. Tindall, E. D. Kwon and R. J. Karnes (2009). "Expression of immunosuppressive B7-H3 ligand by hormone-treated prostate cancer tumors and metastases." Clin Cancer Res **15**(6): 2174-2180.
- Colonna, M. and J. Samaridis (1995). "Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells." Science **268**(5209): 405-408.
- Colonna, M., T. Spies, J. L. Strominger, E. Ciccone, A. Moretta, L. Moretta, D. Pende and O. Viale (1992). "Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene." Proc Natl Acad Sci U S A **89**(17): 7983-7985.
- Coppin, C., F. Porzolt, A. Awa, J. Kumpf, A. Coldman and T. Wilt (2005). "Immunotherapy for advanced renal cell cancer." Cochrane Database Syst Rev(1): CD001425.
- de Kruijf, E. M., A. Sajet, J. G. van Nes, H. Putter, V. T. Smit, R. A. Eagle, I. Jafferji, J. Trowsdale, G. J. Liefers, C. J. van de Velde and P. J. Kuppen (2012). "NKG2D ligand tumor expression and association with clinical outcome in early breast cancer patients: an observational study." BMC Cancer **12**: 24.
- de Rham, C., S. Ferrari-Lacraz, S. Jendly, G. Schneiter, J. M. Dayer and J. Villard (2007). "The proinflammatory cytokines IL-2, IL-15 and IL-21 modulate the repertoire of mature human natural killer cell receptors." Arthritis Res Ther **9**(6): R125.
- Dearnaley, D. P., V. S. Khoo, A. R. Norman, L. Meyer, A. Nahum, D. Tait, J. Yarnold and A. Horwich (1999). "Comparison of radiation side-effects of conformal and conventional radiotherapy in prostate cancer: a randomised trial." Lancet **353**(9149): 267-272.
- Dong, H., S. E. Strome, D. R. Salomao, H. Tamura, F. Hirano, D. B. Flies, P. C. Roche, J. Lu, G. Zhu, K. Tamada, V. A. Lennon, E. Celis and L. Chen (2002). "Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion." Nat Med **8**(8): 793-800.
- Dobrovina, E. S., M. M. Dobrovin, E. Vider, R. B. Sisson, R. J. O'Reilly, B. Dupont and Y. M. Vyas (2003). "Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma." J Immunol **171**(12): 6891-6899.
- Dunn, G. P., C. M. Koebel and R. D. Schreiber (2006). "Interferons, immunity and cancer immunoediting." Nat Rev Immunol **6**(11): 836-848.

Dunn, G. P., L. J. Old and R. D. Schreiber (2004). "The Immunobiology of Cancer Immunosurveillance and Immunoediting." Immunity **21**(2): 137-148.

Ebelt, K., G. Babaryka, B. Frankenberger, C. G. Stief, W. Eisenmenger, T. Kirchner, D. J. Schendel and E. Noessner (2009). "Prostate cancer lesions are surrounded by FOXP3+, PD-1+ and B7-H1+ lymphocyte clusters." Eur J Cancer **45**(9): 1664-1672.

Edge, S., D. Byrd and C. Compton (2010). AJCC Cancer Staging Manual. Prostate. New York, NY, Springer: 457-468.

Ehrlich, P. (1906). Collected Studies in Immunity. New York, John Wiley and Sons.

Elhage, O., C. Galustian, R. Smith and P. Dasgupta (2012). ABC of Prostate Cancer: Immunology of Prostate Cancer, Blackwell Publishing Ltd.

Elhage, O., C. Galustian, R. A. G. Smith and P. Dasgupta (2012). "IL-15 potentiates CD8 and NK effector cell expansion and tumour cell killing in the prostate cancer microenvironment." European Urology Supplements **11**(1): E243-E243.

Elhage, O., C. Galustian, O. Ukimura, I. Gill, R. Smith and P. Dasgupta (2011). "Selection of optimal cytokine combinations for immunotherapy of prostate cancer." Journal of Urology **185**: 719.

Elhage, O., C. Sakellariou, R. A. Smith, C. Galustian and P. Dasgupta (2015). "CD8 T cells inhibit the Interleukin-15 (IL-15) induced cytotoxic activity of NK and NKT cells toward tumour cells in the prostate cancer microenvironment." The Journal of Urology **193**(4): 550.

Epstein, J. I., M. J. Zelefsky, D. D. Sjoberg, J. B. Nelson, L. Egevad, C. Magi-Galluzzi, A. J. Vickers, A. V. Parwani, V. E. Reuter, S. W. Fine, J. A. Eastham, P. Wiklund, M. Han, C. A. Reddy, J. P. Ciezki, T. Nyberg and E. A. Klein (2016). "A Contemporary Prostate Cancer Grading System: A Validated Alternative to the Gleason Score." Eur Urol **69**(3): 428-435.

Fehniger, T. A., M. A. Cooper and M. A. Caligiuri (2002). "Interleukin-2 and interleukin-15: immunotherapy for cancer." Cytokine & Growth Factor Reviews **13**(2): 169-183.

Fehniger, T. A., K. Suzuki, J. B. VanDeusen, M. A. Cooper, A. G. Freud and M. A. Caligiuri (2001). "Fatal leukemia in interleukin-15 transgenic mice." Blood Cells Mol Dis **27**(1): 223-230.

Felio, K., H. Nguyen, C. C. Dascher, H. J. Choi, S. Li, M. I. Zimmer, A. Colmone, D. B. Moody, M. B. Brenner and C. R. Wang (2009). "CD1-restricted adaptive immune responses to Mycobacteria in human group 1 CD1 transgenic mice." J Exp Med **206**(11): 2497-2509.

Fong, L., S. S. Kwek, S. O'Brien, B. Kavanagh, D. G. McNeel, V. Weinberg, A. M. Lin, J. Rosenberg, C. J. Ryan, B. I. Rini and E. J. Small (2009). "Potentiating endogenous antitumor immunity to prostate cancer through combination immunotherapy with CTLA4 blockade and GM-CSF." Cancer Res **69**(2): 609-615.

Foster, B. A., J. R. Gingrich, E. D. Kwon, C. Madias and N. M. Greenberg (1997). "Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model." Cancer Res **57**(16): 3325-3330.

Freeman, G. J., A. J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L. J. Fitz, N. Malenkovich, T. Okazaki, M. C. Byrne, H. F. Horton, L. Fouser, L. Carter, V. Ling, M. R. Bowman, B. M. Carreno, M. Collins, C. R. Wood and T. Honjo (2000). "Engagement of the PD-1 immunoinhibitory receptor by a



novel B7 family member leads to negative regulation of lymphocyte activation." J Exp Med **192**(7): 1027-1034.

Gabrilovich, D. I., S. Ostrand-Rosenberg and V. Bronte (2012). "Coordinated regulation of myeloid cells by tumours." Nat Rev Immunol **12**(4): 253-268.

Gaffen, S. L. and K. D. Liu (2004). "Overview of interleukin-2 function, production and clinical applications." Cytokine **28**(3): 109-123.

Garon, E. B. (2015). "Current Perspectives in Immunotherapy for Non-Small Cell Lung Cancer." Semin Oncol **42 Suppl 2**: S11-18.

Ghiringhelli, F., C. Menard, M. Terme, C. Flament, J. Taieb, N. Chaput, P. E. Puig, S. Novault, B. Escudier, E. Vivier, A. Lécésne, C. Robert, J. Y. Blay, J. Bernard, S. Caillat-Zucman, A. Freitas, T. Tursz, O. Wagner-Ballon, C. Capron, W. Vainchencker, F. Martin and L. Zitvogel (2005). "CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner." J Exp Med **202**(8): 1075-1085.

Gillis, S. and K. A. Smith (1977). "Long term culture of tumour-specific cytotoxic T cells." Nature **268**(5616): 154-156.

Godfrey, D. I., H. R. MacDonald, M. Kronenberg, M. J. Smyth and L. Van Kaer (2004). "NKT cells: what's in a name?" Nat Rev Immunol **4**(3): 231-237.

Gorer, P. A. (1950). "Studies in antibody response of mice to tumour inoculation." Br J Cancer **4**(4): 372-379.

Gravisaco, M. J., C. Mongini, E. Alvarez, P. Ruybal, A. Escalada, M. Sanchez-Lockhart, S. Hajos and C. Waldner (2003). "IL-2, IL-10, IL-15 and TNF are key regulators of murine T-cell lymphoma growth." Int J Mol Med **12**(4): 627-632.

Greenberg, N. M. (1996). "Transgenic models for prostate cancer research." Urol Oncol **2**(4): 119-122.

Gregor, P. D., J. D. Wolchok, C. R. Ferrone, H. Buchinshky, J. A. Guevara-Patino, M. A. Perales, F. Mortazavi, D. Bacich, W. Heston, J. B. Latouche, M. Sadelain, J. P. Allison, H. I. Scher and A. N. Houghton (2004). "CTLA-4 blockade in combination with xenogeneic DNA vaccines enhances T-cell responses, tumor immunity and autoimmunity to self antigens in animal and cellular model systems." Vaccine **22**(13-14): 1700-1708.

Groh, V., R. Rhinehart, H. Secrist, S. Bauer, K. H. Grabstein and T. Spies (1999). "Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB." Proc Natl Acad Sci U S A **96**(12): 6879-6884.

Groh, V., J. Wu, C. Yee and T. Spies (2002). "Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation." Nature **419**(6908): 734-738.

Guerra, N., Y. X. Tan, N. T. Joncker, A. Choy, F. Gallardo, N. Xiong, S. Knoblaugh, D. Cado, N. M. Greenberg and D. H. Raulet (2008). "NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy." Immunity **28**(4): 571-580.

Hadaschik, B., Y. Su, E. Huter, Y. Ge, M. Hohenfellner and P. Beckhove (2012). "Antigen specific T-cell responses against tumor antigens are controlled by regulatory T cells in patients with prostate cancer." J Urol **187**(4): 1458-1465.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Heinlein, C. A. and C. Chang (2002). "Androgen receptor (AR) coregulators: an overview." Endocr Rev **23**(2): 175-200.

Heinlein, C. A. and C. Chang (2002). "The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions." Mol Endocrinol **16**(10): 2181-2187.

Herberman, R. B. and J. R. Ortaldo (1981). "Natural killer cells: their roles in defenses against disease." Science **214**(4516): 24-30.

Higano, C. S., J. M. Corman, D. C. Smith, A. S. Centeno, C. P. Steidle, M. Gittleman, J. W. Simons, N. Sacks, J. Aimi and E. J. Small (2008). "Phase 1/2 dose-escalation study of a GM-CSF-secreting, allogeneic, cellular immunotherapy for metastatic hormone-refractory prostate cancer." Cancer **113**(5): 975-984.

Hinrichs, C. S., R. Spolski, C. M. Paulos, L. Gattinoni, K. W. Kerstann, D. C. Palmer, C. A. Klebanoff, S. A. Rosenberg, W. J. Leonard and N. P. Restifo (2008). "IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy." Blood **111**(11): 5326-5333.

Hodi, F. S., S. J. O'Day, D. F. McDermott, R. W. Weber, J. A. Sosman, J. B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J. C. Hassel, W. Akerley, A. J. van den Eertwegh, J. Lutzky, P. Lorigan, J. M. Vaubel, G. P. Linette, D. Hogg, C. H. Ottensmeier, C. Lebbe, C. Peschel, I. Quirt, J. I. Clark, J. D. Wolchok, J. S. Weber, J. Tian, M. J. Yellin, G. M. Nichol, A. Hoos and W. J. Urba (2010). "Improved survival with ipilimumab in patients with metastatic melanoma." N Engl J Med **363**(8): 711-723.

Horoszewicz, J. S., S. S. Leong, T. M. Chu, Z. L. Wajsman, M. Friedman, L. Papsidero, U. Kim, L. S. Chai, S. Kakati, S. K. Arya and A. A. Sandberg (1980). "The LNCaP cell line--a new model for studies on human prostatic carcinoma." Prog Clin Biol Res **37**: 115-132.

Hou, D. Y., A. J. Muller, M. D. Sharma, J. DuHadaway, T. Banerjee, M. Johnson, A. L. Mellor, G. C. Prendergast and D. H. Munn (2007). "Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses." Cancer Res **67**(2): 792-801.

Hromadnikova, I., S. Li, K. Kotlabova and A. M. Dickinson (2016). "Influence of In Vitro IL-2 or IL-15 Alone or in Combination with Hsp 70 Derived 14-Mer Peptide (TKD) on the Expression of NK Cell Activatory and Inhibitory Receptors on Peripheral Blood T Cells, B Cells and NKT Cells." PLoS One **11**(3): e0151535.

Huntington, N. D., N. Legrand, N. L. Alves, B. Jaron, K. Weijer, A. Plet, E. Corcuff, E. Mortier, Y. Jacques, H. Spits and J. P. Di Santo (2009). "IL-15 trans-presentation promotes human NK cell development and differentiation in vivo." J Exp Med **206**(1): 25-34.

Inoue, A., K. Yamazaki, M. Maemondo, T. Suzuki, Y. Kimura, M. Kanbe, H. Isobe, M. Nishimura, Y. Saijo and T. Nukiwa (2006). "A phase I study of amrubicin combined with carboplatin for elderly patients with small-cell lung cancer." J Thorac.Oncol **1**(6): 551-555.

Jemal, A., F. Bray, M. M. Center, J. Ferlay, E. Ward and D. Forman (2011). "Global cancer statistics." CA Cancer J Clin **61**(2): 69-90.

Jinushi, M., F. S. Hodi and G. Dranoff (2006). "Therapy-induced antibodies to MHC class I chain-related protein A antagonize immune suppression and stimulate antitumor cytotoxicity." Proc Natl Acad Sci U S A **103**(24): 9190-9195.

Jinushi, M., T. Takehara, T. Kanto, T. Tatsumi, V. Groh, T. Spies, T. Miyagi, T. Suzuki, Y. Sasaki and N. Hayashi (2003). "Critical role of MHC class I-related chain A and B expression on IFN- $\alpha$ -stimulated dendritic cells in NK cell activation: impairment in chronic hepatitis C virus infection." J Immunol **170**(3): 1249-1256.

Jinushi, M., T. Takehara, T. Tatsumi, N. Hiramatsu, R. Sakamori, S. Yamaguchi and N. Hayashi (2005). "Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class I-related chain A in advanced human hepatocellular carcinomas." J Hepatol **43**(6): 1013-1020.

Johnson, E. E., H. D. Lum, A. L. Rakhmilevich, B. E. Schmidt, M. Furlong, I. N. Buhtoiarov, J. A. Hank, A. Raubitschek, D. Colcher, R. A. Reisfeld, S. D. Gillies and P. M. Sondel (2008). "Intratumoral immunocytokine treatment results in enhanced antitumor effects." Cancer Immunol Immunother **57**(12): 1891-1902.

Kaighn, M. E., K. S. Narayan, Y. Ohnuki, J. F. Lechner and L. W. Jones (1979). "Establishment and characterization of a human prostatic carcinoma cell line (PC-3)." Invest Urol. **17**(1): 16-23.

Karre, K. (2002). "NK cells, MHC class I molecules and the missing self." Scand.J.Immunol. **55**(3): 221-228.

Kastelan, M., I. Kraljic and M. Tarle (1992). "NK cell activity in treated prostate cancer patients as a probe for circulating tumor cells: hormone regulatory effects in vivo." Prostate **21**(2): 111-120.

Katayama, A., M. Takahara, K. Kishibe, T. Nagato, I. Kunibe, A. Katada, T. Hayashi and Y. Harabuchi (2011). "Expression of B7-H3 in hypopharyngeal squamous cell carcinoma as a predictive indicator for tumor metastasis and prognosis." Int J Oncol **38**(5): 1219-1226.

Kelly, R. W. (1995). "Immunosuppressive mechanisms in semen: implications for contraception." Hum Reprod **10**(7): 1686-1693.

Kiessling, R., E. Klein, H. Pross and H. Wigzell (1975). ""Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell." Eur J Immunol **5**(2): 117-121.

Kilinc, M. O., K. S. Aulakh, R. E. Nair, S. A. Jones, P. Alard, M. M. Kosiewicz and N. K. Egilmez (2006). "Reversing tumor immune suppression with intratumoral IL-12: activation of tumor-associated T effector/memory cells, induction of T suppressor apoptosis, and infiltration of CD8+ T effectors." J Immunol **177**(10): 6962-6973.

Kim, M. H., J. Chung, J. W. Yang, S. M. Chung, N. H. Kwag and J. S. Yoo (2003). "Hydrogen peroxide-induced cell death in a human retinal pigment epithelial cell line, ARPE-19." Korean J Ophthalmol **17**(1): 19-28.

Kiniwa, Y., Y. Miyahara, H. Y. Wang, W. Peng, G. Peng, T. M. Wheeler, T. C. Thompson, L. J. Old and R. F. Wang (2007). "CD8+ Foxp3+ Regulatory T Cells Mediate Immunosuppression in Prostate Cancer." Clinical Cancer Research **13**(23): 6947-6958.

Klapper, J. A., S. G. Downey, F. O. Smith, J. C. Yang, M. S. Hughes, U. S. Kammula, R. M. Sherry, R. E. Royal, S. M. Steinberg and S. Rosenberg (2008). "High-dose interleukin-2 for the treatment of metastatic renal cell carcinoma : a retrospective analysis of response and survival in patients treated in the surgery branch at the National Cancer Institute between 1986 and 2006." Cancer **113**(2): 293-301.

Koo, K. C., D. H. Shim, C. M. Yang, S. B. Lee, S. M. Kim, T. Y. Shin, K. H. Kim, H. G. Yoon, K. H. Rha, J. M. Lee and S. J. Hong (2013). "Reduction of the CD16(-)CD56bright NK cell subset precedes NK cell dysfunction in prostate cancer." PLoS One **8**(11): e78049.

Kreymborg, K., S. Haak, R. Murali, J. Wei, R. Waitz, G. Gasteiger, P. A. Savage, M. R. van den Brink and J. P. Allison (2015). "Ablation of B7-H3 but Not B7-H4 Results in Highly Increased Tumor Burden in a Murine Model of Spontaneous Prostate Cancer." Cancer Immunol Res **3**(8): 849-854.

Kritikou, J. S., C. I. Dahlberg, M. A. Baptista, A. K. Wagner, P. P. Banerjee, L. A. Gwalani, C. Poli, S. K. Panda, K. Karre, S. M. Kaech, F. Wermeling, J. Andersson, J. S. Orange, H. Brauner and L. S. Westerberg (2016). "IL-2 in the tumor microenvironment is necessary for Wiskott-Aldrich syndrome protein deficient NK cells to respond to tumors in vivo." Sci Rep **6**: 30636.

Kuhl, A. A., N. N. Pawlowski, K. Grollich, M. Bleszenohl, J. Westermann, M. Zeitz, C. Loddenkemper and J. C. Hoffmann (2009). "Human peripheral gammadelta T cells possess regulatory potential." Immunology **128**(4): 580-588.

Kwon, E. D., C. G. Drake, H. I. Scher, K. Fizazi, A. Bossi, A. J. van den Eertwegh, M. Krainer, N. Houede, R. Santos, H. Mahammedi, S. Ng, M. Maio, F. A. Franke, S. Sundar, N. Agarwal, A. M. Bergman, T. E. Ciuleanu, E. Korbenfeld, L. Sengelov, S. Hansen, C. Logothetis, T. M. Beer, M. B. McHenry, P. Gagnier, D. Liu, W. R. Gerritsen and C. A. Investigators (2014). "Ipilimumab versus placebo after radiotherapy in patients with metastatic castration-resistant prostate cancer that had progressed after docetaxel chemotherapy (CA184-043): a multicentre, randomised, double-blind, phase 3 trial." Lancet Oncol **15**(7): 700-712.

Kwon, E. D., B. A. Foster, A. A. Hurwitz, C. Madias, J. P. Allison, N. M. Greenberg and M. B. Burg (1999). "Elimination of residual metastatic prostate cancer after surgery and adjunctive cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade immunotherapy." Proc Natl Acad Sci U S A **96**(26): 15074-15079.

Kwon, E. D., B. A. Foster, A. A. Hurwitz, C. Madias, J. P. Allison, N. M. Greenberg and M. B. Burg (1999). "Elimination of residual metastatic prostate cancer after surgery and adjunctive cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade immunotherapy." Proc.Natl.Acad.Sci.U.S.A **96**(26): 15074-15079.

Lanier, L. L. (2005). "NK cell recognition." Annu Rev Immunol **23**: 225-274.

Lanier, L. L. and J. H. Phillips (1996). "Inhibitory MHC class I receptors on NK cells and T cells." Immunol Today **17**(2): 86-91.

Lebbe, C., J. S. Weber, M. Maio, B. Neyns, K. Harmankaya, O. Hamid, S. J. O'Day, C. Konto, L. Cykowski, M. B. McHenry and J. D. Wolchok (2014). "Survival follow-up and ipilimumab retreatment

of patients with advanced melanoma who received ipilimumab in prior phase II studies." Ann Oncol **25**(11): 2277-2284.

Lissoni, P., F. Rovelli, M. Mandala and S. Barni (1998). "Blood concentrations of interleukin-15 in cancer patients and their variations during interleukin-2 immunotherapy: preliminary considerations." Int J Biol Markers **13**(3): 169-171.

Little, R. F., J. M. Pluda, K. M. Wyvill, I. R. Rodriguez-Chavez, G. Tosato, A. T. Catanzaro, S. M. Steinberg and R. Yarchoan (2006). "Activity of subcutaneous interleukin-12 in AIDS-related Kaposi sarcoma." Blood **107**(12): 4650-4657.

Liu, G., S. Lu, X. Wang, S. T. Page, C. S. Higano, S. R. Plymate, N. M. Greenberg, S. Sun, Z. Li and J. D. Wu (2013). "Perturbation of NK cell peripheral homeostasis accelerates prostate carcinoma metastasis." J Clin Invest **123**(10): 4410-4422.

Liu, V. C., L. Y. Wong, T. Jang, A. H. Shah, I. Park, X. Yang, Q. Zhang, S. Lonning, B. A. Teicher and C. Lee (2007). "Tumor evasion of the immune system by converting CD4+CD25- T cells into CD4+CD25+ T regulatory cells: role of tumor-derived TGF-beta." J Immunol **178**(5): 2883-2892.

Liu, Y., L. Vlatkovic, T. Saeter, E. Servoll, G. Waaler, J. M. Nesland, K. E. Giercksky and K. Axcrone (2012). "Is the clinical malignant phenotype of prostate cancer a result of a highly proliferative immune-evasive B7-H3-expressing cell population?" Int J Urol **19**(8): 749-756.

Loberg, R. D., K. J. Wojno, L. L. Day and K. J. Pienta (2005). "Analysis of membrane-bound complement regulatory proteins in prostate cancer." Urology **66**(6): 1321-1326.

Ma, H. L., M. J. Whitters, R. F. Konz, M. Senices, D. A. Young, M. J. Grusby, M. Collins and K. Dunussi-Joannopoulos (2003). "IL-21 activates both innate and adaptive immunity to generate potent antitumor responses that require perforin but are independent of IFN-gamma." J Immunol **171**(2): 608-615.

MacLennan, G. T. (2012). Prostate and Urethral Sphincters. Hinman's Atlas of Urosurgical Anatomy. Philadelphia, PA, Saunders Elsevier.

Malkovsky, M. and P. M. Sondel (1987). "Interleukin 2 and its receptor: structure, function and therapeutic potential." Blood Rev **1**(4): 254-266.

Mandelboim, O., P. Malik, D. M. Davis, C. H. Jo, J. E. Boyson and J. L. Strominger (1999). "Human CD16 as a lysis receptor mediating direct natural killer cell cytotoxicity." Proc Natl Acad Sci U S A **96**(10): 5640-5644.

Marten, A., S. Renoth, M. von Lilienfeld-Toal, P. Buttgerit, F. Schakowski, A. Glasmacher, T. Sauerbruch and I. G. Schmidt-Wolf (2001). "Enhanced lytic activity of cytokine-induced killer cells against multiple myeloma cells after co-culture with idiotype-pulsed dendritic cells." Haematologica **86**(10): 1029-1037.

McLaren, E. (2016) "Deaths registered in England and Wales: 2015."

McNeal J.E., B. D. (1990). Anatomy of the prostate: implications for disease. Pathology of the Prostate. New York, Churchill Livingstone. **2**.

McNeal, J. E. (1981). "The zonal anatomy of the prostate." Prostate **2**(1): 35-49.

McNeel, D. G., H. A. Smith, J. C. Eickhoff, J. M. Lang, M. J. Staab, G. Wilding and G. Liu (2012). "Phase I trial of tremelimumab in combination with short-term androgen deprivation in patients with PSA-recurrent prostate cancer." Cancer Immunol Immunother **61**(7): 1137-1147.

Meinhardt, K., I. Kroeger, A. Abendroth, S. Muller, A. Mackensen and E. Ullrich (2012). "Influence of NK cell magnetic bead isolation methods on phenotype and function of murine NK cells." J Immunol Methods **378**(1-2): 1-10.

Mention, J. J., M. Ben Ahmed, B. Begue, U. Barbe, V. Verkarre, V. Asnafi, J. F. Colombel, P. H. Cugnenc, F. M. Ruemmele, E. McIntyre, N. Brousse, C. Cellier and N. Cerf-Bensussan (2003). "Interleukin 15: a key to disrupted intraepithelial lymphocyte homeostasis and lymphomagenesis in celiac disease." Gastroenterology **125**(3): 730-745.

Meresse, B., Z. Chen, C. Ciszewski, M. Tretiakova, G. Bhagat, T. N. Krausz, D. H. Raulet, L. L. Lanier, V. Groh, T. Spies, E. C. Ebert, P. H. Green and B. Jabri (2004). "Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease." Immunity **21**(3): 357-366.

Metelitsa, L. S., O. V. Naidenko, A. Kant, H. W. Wu, M. J. Loza, B. Perussia, M. Kronenberg and R. C. Seeger (2001). "Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells." J Immunol **167**(6): 3114-3122.

Mocellin, S., S. Pasquali, C. R. Rossi and D. Nitti (2010). "Interferon alpha adjuvant therapy in patients with high-risk melanoma: a systematic review and meta-analysis." J Natl Cancer Inst **102**(7): 493-501.

Moretta, L. and A. Moretta (2004). "Killer immunoglobulin-like receptors." Curr Opin Immunol **16**(5): 626-633.

Mottet, N., Bellmunt, E.B., van den Bergh, R.C.N., Bolla, M., van Casteren, N.J., Cornford, P., Culine, S., Joniau, S., Lam, T., Mason, M.D., Matveev, V., van der Poel, H., van der Kwast, T.H., Rouviere, O., Wiegel, T. (2015). "Guidelines on Prostate Cancer." European Association of Urology.

Munn, D. H. and A. L. Mellor (2007). "Indoleamine 2,3-dioxygenase and tumor-induced tolerance." J Clin Invest **117**(5): 1147-1154.

Muntasell, A., G. Magri, D. Pende, A. Angulo and M. Lopez-Botet (2010). "Inhibition of NKG2D expression in NK cells by cytokines secreted in response to human cytomegalovirus infection." Blood **115**(25): 5170-5179.

Murphy, R. (2016) "Cancer Registration Statistics, England: 2014."

Muta, T., T. Kurosaki, Z. Misulovin, M. Sanchez, M. C. Nussenzweig and J. V. Ravetch (1994). "A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling." Nature **368**(6466): 70-73.

Negrier, S., B. Escudier, C. Lasset, J. Y. Douillard, J. Savary, C. Chevreau, A. Ravaud, A. Mercatello, J. Peny, M. Mousseau, T. Philip and T. Tursz (1998). "Recombinant human interleukin-2, recombinant human interferon alfa-2a, or both in metastatic renal-cell carcinoma. Groupe Francais d'Immunotherapie." N Engl J Med **338**(18): 1272-1278.

O'Konek, J. J., S. Kato, S. Takao, L. Izhak, Z. Xia, P. Illarionov, G. S. Besra, M. Terabe and J. A. Berzofsky (2013). "beta-mannosylceramide activates type I natural killer t cells to induce tumor immunity without inducing long-term functional anergy." Clin Cancer Res **19**(16): 4404-4411.

O'Sullivan, T., R. Saddawi-Konefka, W. Vermi, C. M. Koebel, C. Arthur, J. M. White, R. Uppaluri, D. M. Andrews, S. F. Ngiow, M. W. Teng, M. J. Smyth, R. D. Schreiber and J. D. Bui (2012). "Cancer immunoediting by the innate immune system in the absence of adaptive immunity." J Exp Med **209**(10): 1869-1882.

Old, L. J., E. A. Boyse and E. Stockert (1965). "The G (Gross) leukemia antigen." Cancer Res **25**(6): 813-819.

Olson, B. M., E. Jankowska-Gan, J. T. Becker, D. A. Vignali, W. J. Burlingham and D. G. McNeel (2012). "Human prostate tumor antigen-specific CD8<sup>+</sup> regulatory T cells are inhibited by CTLA-4 or IL-35 blockade." J Immunol **189**(12): 5590-5601.

Pahl, J. and A. Cerwenka (2015). "Tricking the balance: NK cells in anti-cancer immunity." Immunobiology.

Palucka, K. and J. Banchereau (2012). "Cancer immunotherapy via dendritic cells." Nat Rev Cancer **12**(4): 265-277.

Pardoll, D. M. (2012). "The blockade of immune checkpoints in cancer immunotherapy." Nat Rev Cancer **12**(4): 252-264.

Parekh, H. and B. I. Rini (2015). "Emerging therapeutic approaches in renal cell carcinoma." Expert Rev Anticancer Ther **15**(11): 1305-1314.

Patel, H., R. A. Smith, S. H. Sacks and W. Zhou (2006). "Therapeutic strategy with a membrane-localizing complement regulator to increase the number of usable donor organs after prolonged cold storage." J Am Soc Nephrol **17**(4): 1102-1111.

Pende, D., S. Parolini, A. Pessino, S. Sivori, R. Augugliaro, L. Morelli, E. Marcenaro, L. Accame, A. Malaspina, R. Biassoni, C. Bottino, L. Moretta and A. Moretta (1999). "Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells." J Exp Med **190**(10): 1505-1516.

Piao, Y. R., Z. H. Jin, K. C. Yuan and X. S. Jin (2014). "Analysis of Tim-3 as a therapeutic target in prostate cancer." Tumour Biol **35**(11): 11409-11414.

Prieto, P. A., J. C. Yang, R. M. Sherry, M. S. Hughes, U. S. Kammula, D. E. White, C. L. Levy, S. A. Rosenberg and G. Q. Phan (2012). "CTLA-4 blockade with ipilimumab: long-term follow-up of 177 patients with metastatic melanoma." Clin Cancer Res **18**(7): 2039-2047.

Qian, Y., H. P. Yao, L. Shen, L. F. Cheng and L. H. Zhang (2010). "[Expression of B7-H4 in prostate cancer and its clinical significance]." Zhejiang Da Xue Xue Bao Yi Xue Ban **39**(4): 345-349.

Raffaghello, L., I. Prigione, I. Airoidi, M. Camoriano, I. Levreri, C. Gambini, D. Pende, A. Steinle, S. Ferrone and V. Pistoia (2004). "Downregulation and/or release of NKG2D ligands as immune evasion strategy of human neuroblastoma." Neoplasia **6**(5): 558-568.

Raulet, D. H., S. Gasser, B. G. Gowen, W. Deng and H. Jung (2013). "Regulation of ligands for the NKG2D activating receptor." Annu Rev Immunol **31**: 413-441.

- Rausch, A., M. Hessmann, A. Holscher, T. Schreiber, S. Bulfone-Paus, S. Ehlers and C. Holscher (2006). "Interleukin-15 mediates protection against experimental tuberculosis: a role for NKG2D-dependent effector mechanisms of CD8+ T cells." Eur J Immunol **36**(5): 1156-1167.
- Rei, M., N. Goncalves-Sousa, T. Lanca, R. G. Thompson, S. Mensurado, F. R. Balkwill, H. Kulbe, D. J. Pennington and B. Silva-Santos (2014). "Murine CD27(-) Vgamma6(+) gammadelta T cells producing IL-17A promote ovarian cancer growth via mobilization of protumor small peritoneal macrophages." Proc Natl Acad Sci U S A **111**(34): E3562-3570.
- Rei, M., D. J. Pennington and B. Silva-Santos (2015). "The emerging Protumor role of gammadelta T lymphocytes: implications for cancer immunotherapy." Cancer Res **75**(5): 798-802.
- Reis e Sousa, C. (2001). "Dendritic cells as sensors of infection." Immunity **14**(5): 495-498.
- Reth, M. (1989). "Antigen receptor tail clue." Nature **338**(6214): 383-384.
- Rincon-Orozco, B., V. Kunzmann, P. Wrobel, D. Kabelitz, A. Steinle and T. Herrmann (2005). "Activation of V gamma 9V delta 2 T cells by NKG2D." J Immunol **175**(4): 2144-2151.
- Robert, C., G. V. Long, B. Brady, C. Dutriaux, M. Maio, L. Mortier, J. C. Hassel, P. Rutkowski, C. McNeil, E. Kalinka-Warzocha, K. J. Savage, M. M. Hernberg, C. Lebbe, J. Charles, C. Mihalciou, V. Chiarion-Sileni, C. Mauch, F. Cognetti, A. Arance, H. Schmidt, D. Schadendorf, H. Gogas, L. Lundgren-Eriksson, C. Horak, B. Sharkey, I. M. Waxman, V. Atkinson and P. A. Ascierto (2015). "Nivolumab in previously untreated melanoma without BRAF mutation." N Engl J Med **372**(4): 320-330.
- Roberts, A. I., L. Lee, E. Schwarz, V. Groh, T. Spies, E. C. Ebert and B. Jabri (2001). "NKG2D receptors induced by IL-15 costimulate CD28-negative effector CTL in the tissue microenvironment." J Immunol **167**(10): 5527-5530.
- Romee, R., J. W. Leong and T. A. Fehniger (2014). "Utilizing cytokines to function-enable human NK cells for the immunotherapy of cancer." Scientifica (Cairo) **2014**: 205796.
- Rook, A. H., G. S. Wood, E. K. Yoo, R. Elenitsas, D. M. Kao, M. L. Sherman, W. K. Witmer, K. A. Rockwell, R. B. Shane, S. R. Lessin and E. C. Vonderheid (1999). "Interleukin-12 therapy of cutaneous T-cell lymphoma induces lesion regression and cytotoxic T-cell responses." Blood **94**(3): 902-908.
- Roth, T. J., Y. Sheinin, C. M. Lohse, S. M. Kuntz, X. Frigola, B. A. Inman, A. E. Krambeck, M. E. McKenney, R. J. Karnes, M. L. Blute, J. C. Cheville, T. J. Sebo and E. D. Kwon (2007). "B7-H3 ligand expression by prostate cancer: a novel marker of prognosis and potential target for therapy." Cancer Res **67**(16): 7893-7900.
- Sakuishi, K., L. Apetoh, J. M. Sullivan, B. R. Blazar, V. K. Kuchroo and A. C. Anderson (2010). "Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity." J Exp Med **207**(10): 2187-2194.
- Sakuishi, K., P. Jayaraman, S. M. Behar, A. C. Anderson and V. K. Kuchroo (2011). "Emerging Tim-3 functions in antimicrobial and tumor immunity." Trends Immunol **32**(8): 345-349.
- Sato, N., H. Sabzevari, S. Fu, W. Ju, M. N. Petrus, R. N. Bamford, T. A. Waldmann and Y. Tagaya (2011). "Development of an IL-15-autocrine CD8 T-cell leukemia in IL-15-transgenic mice requires the cis expression of IL-15Ralpha." Blood **117**(15): 4032-4040.



Schellhammer, P. F., G. Chodak, J. B. Whitmore, R. Sims, M. W. Frohlich and P. W. Kantoff (2013). "Lower baseline prostate-specific antigen is associated with a greater overall survival benefit from sipuleucel-T in the Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT) trial." Urology **81**(6): 1297-1302.

Schwartzentruber, D. J., D. H. Lawson, J. M. Richards, R. M. Conry, D. M. Miller, J. Treisman, F. Gailani, L. Riley, K. Conlon, B. Pockaj, K. L. Kendra, R. L. White, R. Gonzalez, T. M. Kuzel, B. Curti, P. D. Leming, E. D. Whitman, J. Balkissoon, D. S. Reintgen, H. Kaufman, F. M. Marincola, M. J. Merino, S. A. Rosenberg, P. Choyke, D. Vena and P. Hwu (2011). "gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma." N Engl J Med **364**(22): 2119-2127.

Seidel, U. J., P. Schlegel and P. Lang (2013). "Natural killer cell mediated antibody-dependent cellular cytotoxicity in tumor immunotherapy with therapeutic antibodies." Front Immunol **4**: 76.

Sfanos, K. S., T. C. Bruno, C. H. Maris, L. Xu, C. J. Thoburn, A. M. DeMarzo, A. K. Meeker, W. B. Isaacs and C. G. Drake (2008). "Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing." Clin Cancer Res **14**(11): 3254-3261.

Shafer-Weaver, K. A., M. J. Anderson, K. Stagliano, A. Malyguine, N. M. Greenberg and A. A. Hurwitz (2009). "Cutting Edge: Tumor-Specific CD8+ T Cells Infiltrating Prostatic Tumors Are Induced to Become Suppressor Cells." The Journal of Immunology **183**(8): 4848-4852.

Shi, Y., X. Fan, W. Meng, H. Deng, N. Zhang and Z. An (2014). "Engagement of immune effector cells by trastuzumab induces HER2/ERBB2 downregulation in cancer cells through STAT1 activation." Breast Cancer Res **16**(2): R33.

Shirota, Y., H. Shirota and D. M. Klinman (2012). "Intratumoral injection of CpG oligonucleotides induces the differentiation and reduces the immunosuppressive activity of myeloid-derived suppressor cells." J Immunol **188**(4): 1592-1599.

Siegel, R., D. Naishadham and A. Jemal (2012). "Cancer statistics, 2012." CA Cancer J Clin **62**(1): 10-29.

Siegel, R. L., K. D. Miller and A. Jemal (2016). "Cancer statistics, 2016." CA Cancer J Clin **66**(1): 7-30.

Sims, R. B. (2012). "Development of sipuleucel-T: autologous cellular immunotherapy for the treatment of metastatic castrate resistant prostate cancer." Vaccine **30**(29): 4394-4397.

Sivori, S., M. Vitale, L. Morelli, L. Sanseverino, R. Augugliaro, C. Bottino, L. Moretta and A. Moretta (1997). "p46, a novel natural killer cell-specific surface molecule that mediates cell activation." J Exp Med **186**(7): 1129-1136.

Small, E. J., P. F. Schellhammer, C. S. Higano, C. H. Redfern, J. J. Nemunaitis, F. H. Valone, S. S. Verjee, L. A. Jones and R. M. Hershberg (2006). "Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer." J Clin Oncol **24**(19): 3089-3094.

Smith, G. P. and R. A. Smith (2001). "Membrane-targeted complement inhibitors." Mol Immunol **38**(2-3): 249-255.

Smith, G. P. and R. A. G. Smith (2001). "Membrane-targeted complement inhibitors." Molecular Immunology **38**(2-3): 249-255.

- Smith, R. A. G. and R. Cassels (1988). "Preparation and properties of a reversible conjugate of immunoglobulin G with the active centre of human tissue plasminogen activator." Fibrinolysis **2**(4): 189-195.
- Smyth, M. J., E. Cretney, M. H. Kershaw and Y. Hayakawa (2004). "Cytokines in cancer immunity and immunotherapy." Immunol Rev **202**: 275-293.
- Soman, G., X. Yang, H. Jiang, S. Giardina, V. Vyas, G. Mitra, J. Yovandich, S. P. Creekmore, T. A. Waldmann, O. Quinones and W. G. Alvord (2009). "MTS dye based colorimetric CTLL-2 cell proliferation assay for product release and stability monitoring of interleukin-15: assay qualification, standardization and statistical analysis." J Immunol Methods **348**(1-2): 83-94.
- Song, H., J. Kim, D. Cosman and I. Choi (2006). "Soluble ULBP suppresses natural killer cell activity via down-regulating NKG2D expression." Cell Immunol **239**(1): 22-30.
- Spary, L. K., J. Salimu, J. P. Webber, A. Clayton, M. D. Mason and Z. Tabi (2014). "Tumor stroma-derived factors skew monocyte to dendritic cell differentiation toward a suppressive CD14<sup>+</sup> PD-L1<sup>+</sup> phenotype in prostate cancer." Oncoimmunology **3**(9): e955331.
- Su, X., J. Ye, E. C. Hsueh, Y. Zhang, D. F. Hoft and G. Peng (2010). "Tumor microenvironments direct the recruitment and expansion of human Th17 cells." J Immunol **184**(3): 1630-1641.
- Sullivan, R. J. and K. T. Flaherty (2015). "Pembrolizumab for Treatment of Patients with Advanced or Unresectable Melanoma." Clin Cancer Res **21**(13): 2892-2897.
- Sungur, C. M. and W. J. Murphy (2014). "Positive and negative regulation by NK cells in cancer." Crit Rev Oncog **19**(1-2): 57-66.
- Sutherland, C. L., N. J. Chalupny, K. Schooley, T. VandenBos, M. Kubin and D. Cosman (2002). "UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells." J Immunol **168**(2): 671-679.
- Sutherland, C. L., B. Rabinovich, N. J. Chalupny, P. Brawand, R. Miller and D. Cosman (2006). "ULBPs, human ligands of the NKG2D receptor, stimulate tumor immunity with enhancement by IL-15." Blood **108**(4): 1313-1319.
- Szczepanski, M. J., M. Szajnik, A. Welsh, K. A. Foon, T. L. Whiteside and M. Boyiadzis (2010). "Interleukin-15 enhances natural killer cell cytotoxicity in patients with acute myeloid leukemia by upregulating the activating NK cell receptors." Cancer Immunol Immunother **59**(1): 73-79.
- Tagaya, Y., R. N. Bamford, A. P. DeFilippis and T. A. Waldmann (1996). "IL-15: a pleiotropic cytokine with diverse receptor/signaling pathways whose expression is controlled at multiple levels." Immunity **4**(4): 329-336.
- Tekle, C., M. K. Nygren, Y. W. Chen, I. Dybsjord, J. M. Nesland, G. M. Maelandsmo and O. Fodstad (2012). "B7-H3 contributes to the metastatic capacity of melanoma cells by modulation of known metastasis-associated genes." Int J Cancer **130**(10): 2282-2290.
- Topalian, S. L., C. G. Drake and D. M. Pardoll (2012). "Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity." Curr Opin Immunol **24**(2): 207-212.
- Topalian, S. L., F. S. Hodi, J. R. Brahmer, S. N. Gettinger, D. C. Smith, D. F. McDermott, J. D. Powderly, R. D. Carvajal, J. A. Sosman, M. B. Atkins, P. D. Leming, D. R. Spigel, S. J. Antonia, L. Horn, C. G. Drake,

D. M. Pardoll, L. Chen, W. H. Sharfman, R. A. Anders, J. M. Taube, T. L. McMiller, H. Xu, A. J. Korman, M. Jure-Kunkel, S. Agrawal, D. McDonald, G. D. Kollia, A. Gupta, J. M. Wigginton and M. Sznol (2012). "Safety, activity, and immune correlates of anti-PD-1 antibody in cancer." N Engl J Med **366**(26): 2443-2454.

Topham, N. J. and E. W. Hewitt (2009). "Natural killer cell cytotoxicity: how do they pull the trigger?" Immunology **128**(1): 7-15.

Toura, I., T. Kawano, Y. Akutsu, T. Nakayama, T. Ochiai and M. Taniguchi (1999). "Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide." J Immunol **163**(5): 2387-2391.

Tse, B. W., A. Collins, M. K. Oehler, A. Zippelius and V. A. Heinzelmann-Schwarz (2014). "Antibody-based immunotherapy for ovarian cancer: where are we at?" Ann Oncol **25**(2): 322-331.

Ugai, S., O. Shimozaoto, L. Yu, Y. Q. Wang, K. Kawamura, H. Yamamoto, T. Yamaguchi, H. Saisho, S. Sakiyama and M. Tagawa (2003). "Transduction of the IL-21 and IL-23 genes in human pancreatic carcinoma cells produces natural killer cell-dependent and -independent antitumor effects." Cancer Gene Ther **10**(10): 771-778.

Vitale, M., C. Bottino, S. Sivori, L. Sanseverino, R. Castriconi, E. Marcenaro, R. Augugliaro, L. Moretta and A. Moretta (1998). "NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis." J Exp Med **187**(12): 2065-2072.

Vivier, E., E. Tomasello, M. Baratin, T. Walzer and S. Ugolini (2008). "Functions of natural killer cells." Nat Immunol **9**(5): 503-510.

Vivier, E., E. Tomasello, M. Baratin, T. Walzer and S. Ugolini (2008). "Functions of natural killer cells." Nat Immunol **9**(5): 503-510.

Voskoboinik, I., M. J. Smyth and J. A. Trapani (2006). "Perforin-mediated target-cell death and immune homeostasis." Nat Rev Immunol **6**(12): 940-952.

Waldhauer, I., D. Goehlsdorf, F. Gieseke, T. Weinschenk, M. Wittenbrink, A. Ludwig, S. Stevanovic, H. G. Rammensee and A. Steinle (2008). "Tumor-associated MICA is shed by ADAM proteases." Cancer Res **68**(15): 6368-6376.

Waldhauer, I. and A. Steinle (2006). "Proteolytic release of soluble UL16-binding protein 2 from tumor cells." Cancer Res **66**(5): 2520-2526.

Walzer, T., M. Dalod, E. Vivier and L. Zitvogel (2005). "Natural killer cell-dendritic cell crosstalk in the initiation of immune responses." Expert Opin Biol Ther **5 Suppl 1**: S49-59.

Wang, W., A. K. Erbe, J. A. Hank, Z. S. Morris and P. M. Sondel (2015). "NK Cell-Mediated Antibody-Dependent Cellular Cytotoxicity in Cancer Immunotherapy." Front Immunol **6**: 368.

Weber, J. S., S. P. D'Angelo, D. Minor, F. S. Hodi, R. Gutzmer, B. Neyns, C. Hoeller, N. I. Khushalani, W. H. Miller, Jr., C. D. Lao, G. P. Linette, L. Thomas, P. Lorigan, K. F. Grossmann, J. C. Hassel, M. Maio, M. Sznol, P. A. Ascierto, P. Mohr, B. Chmielowski, A. Bryce, I. M. Svane, J. J. Grob, A. M. Krackhardt, C. Horak, A. Lambert, A. S. Yang and J. Larkin (2015). "Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial." Lancet Oncol **16**(4): 375-384.

- Wein, K., Novick, Partin, Peters (2012). *Anatomy of the lower Urinary Tract and Male Genitalia. Campbell-Walsh Urology*. Philadelphia, PA, Saunders Elsevier.
- Willmsky, G., M. Czeh, C. Loddenkemper, J. Gellermann, K. Schmidt, P. Wust, H. Stein and T. Blankenstein (2008). "Immunogenicity of premalignant lesions is the primary cause of general cytotoxic T lymphocyte unresponsiveness." *J Exp Med* **205**(7): 1687-1700.
- Wing, K., T. Yamaguchi and S. Sakaguchi (2011). "Cell-autonomous and -non-autonomous roles of CTLA-4 in immune regulation." *Trends Immunol* **32**(9): 428-433.
- Witte, T., K. Wordelmann and R. E. Schmidt (1990). "Heterogeneity of human natural killer cells in the spleen." *Immunology* **69**(1): 166-170.
- Wu, C. P., J. T. Jiang, M. Tan, Y. B. Zhu, M. Ji, K. F. Xu, J. M. Zhao, G. B. Zhang and X. G. Zhang (2006). "Relationship between co-stimulatory molecule B7-H3 expression and gastric carcinoma histology and prognosis." *World J Gastroenterol* **12**(3): 457-459.
- Wu, J. (2016). "Antibody targeting soluble NKG2D ligand sMIC refuels and invigorates the endogenous immune system to fight cancer." *Oncoimmunology* **5**(3): e1095434.
- Wu, J., Y. Song, A. B. Bakker, S. Bauer, T. Spies, L. L. Lanier and J. H. Phillips (1999). "An activating immunoreceptor complex formed by NKG2D and DAP10." *Science* **285**(5428): 730-732.
- Wu, J. D., L. M. Higgins, A. Steinle, D. Cosman, K. Haugk and S. R. Plymate (2004). "Prevalent expression of the immunostimulatory MHC class I chain-related molecule is counteracted by shedding in prostate cancer." *J Clin Invest* **114**(4): 560-568.
- Yokoyama, W. M., S. Kim and A. R. French (2004). "The dynamic life of natural killer cells." *Annu Rev Immunol* **22**: 405-429.
- Younes, A., B. Pro, M. J. Robertson, I. W. Flinn, J. E. Romaguera, F. Hagemeister, N. H. Dang, P. Fiumara, E. M. Loyer, F. F. Cabanillas, P. W. McLaughlin, M. A. Rodriguez and F. Samaniego (2004). "Phase II clinical trial of interleukin-12 in patients with relapsed and refractory non-Hodgkin's lymphoma and Hodgkin's disease." *Clin Cancer Res* **10**(16): 5432-5438.
- Yu, P., J. C. Steel, M. Zhang, J. C. Morris, R. Waitz, M. Fassio, J. P. Allison and T. A. Waldmann (2012). "Simultaneous inhibition of two regulatory T-cell subsets enhanced Interleukin-15 efficacy in a prostate tumor model." *Proceedings of the National Academy of Sciences*.
- Zang, X. and J. P. Allison (2007). "The B7 family and cancer therapy: costimulation and coinhibition." *Clin Cancer Res* **13**(18 Pt 1): 5271-5279.
- Zang, X., R. H. Thompson, H. A. Al-Ahmadie, A. M. Serio, V. E. Reuter, J. A. Eastham, P. T. Scardino, P. Sharma and J. P. Allison (2007). "B7-H3 and B7x are highly expressed in human prostate cancer and associated with disease spread and poor outcome." *Proc Natl Acad Sci U S A* **104**(49): 19458-19463.
- Zhang, J., F. Basher and J. D. Wu (2015). "NKG2D Ligands in Tumor Immunity: Two Sides of a Coin." *Front Immunol* **6**: 97.
- Zhang, Q., X. J. Yang, S. D. Kundu, M. Pins, B. Javonovic, R. Meyer, S. J. Kim, N. M. Greenberg, T. Kuzel, R. Meagher, Y. Guo and C. Lee (2006). "Blockade of transforming growth factor- $\beta$  signaling in tumor-reactive CD8(+) T cells activates the antitumor immune response cycle." *Mol Cancer Ther* **5**(7): 1733-1743.

Zhao, J., J. Zhao and S. Perlman (2012). "Differential effects of IL-12 on Tregs and non-Treg T cells: roles of IFN-gamma, IL-2 and IL-2R." PLoS One **7**(9): e46241.

Zou, W. and L. Chen (2008). "Inhibitory B7-family molecules in the tumour microenvironment." Nat Rev Immunol **8**(6): 467-477.

Zwirner, N. W. and C. I. Domaica (2010). "Cytokine regulation of natural killer cell effector functions." Biofactors **36**(4): 274-288.